INSECTICIDES – ADVANCES IN INTEGRATED PEST MANAGEMENT

Edited by **Farzana Perveen**

INTECHWEB.ORG

Insecticides – Advances in Integrated Pest Management
Edited by Farzana Perveen

Published by InTech
Janeza Trdine 9, 51000 Rijeka, Croatia

Publishing Process Manager Dejan Grgur
Technical Editor Teodora Smiljanic
Cover Designer InTech Design Team
Image Copyright Roxana, 2011. DepositPhotos

First published December, 2011
Printed in Croatia

A free online edition of this book is available at www.intechopen.com
Additional hard copies can be obtained from orders@intechweb.org

Insecticides – Advances in Integrated Pest Management, Edited by Farzana Perveen
p. cm.
ISBN 978-953-307-780-2

Contents

Preface

Globally, the production quantities of agricultural commodities are increasingly fluctuating, and crop yields are low in relation to the needs of the world population. Furthermore, arthropod pests destroy 20-30% of the world's food supply every year. They damage agricultural crops and harvested food, as well as transmit diseases to humans and animals. Stored-product insects infest raw grain, processed cereals, warehouses, and flour and feed mills. The presence of insects in commodities or structures leads to quantitative and qualitative losses of grain and processed food.

The science applied for the detection of damage is known as Forensic Science. Various analytical methods, i.e. gas chromatography, high performance liquid chromatography, spectrophotometry, polarography, fluorimetry and mass spectrometry are used for detection and determination of residues of different pesticides and drugs involved in forensic work. Arthropods are considered to be a global health threat since they are responsible for transmission of several new and re-emerging human diseases, such as malaria, dengue and yellow fever, Lyme disease, ehrlichiosis and tularemia. Vector-borne human and veterinary diseases, in which pest species function as vectors for transmission are of increasing concern to the general population, more specifically, to the public health. They present a significant threat to the productivity, health, normal lifecycle of humans, livestock, domestic animals and wildlife.

Agricultural production resorts to the use of a varied and large quantity of insecticides to improve the production and preservation of foodstuff. Thus, the use of insecticides has increased rapidly and is now widespread. Beneficial insects like parasitoids, pathogens, predators and pollinators have gained significance. IPM programs have demonstrated that current levels of pesticide use are not necessary in many situations, and are frequently even counter-productive. Excessive and otherwise inappropriate pesticide use is an unnecessary burden on a farmer's health and income, as well as on public health and the environment. Ecological modeling is an important tool for systematic study of the use of IPM technique to control insect populations. Different scenarios can be planned and tested prior to implementation, making experimental designs more efficient and saving time and money. Pyrethroids should be used with caution as insecticidal formulas; they can impair memory and movement in non-target animals. The use of aerosol technology for insect control in food-storage and food-

processing facilities is gaining popularity as a viable alternative to expensive methods of insect disinfestation, such as fumigation and heat treatment. It has several advantages over other methods of insect disinfestations.

In this book, an effort has been made to pool the information on various aspects of pests, vectors, pesticides, parasitoids, predators and resistance. This book can be of use to researchers, scientists, students and farmers.

Farzana Perveen
Chairperson, Department of Zoology
Hazara University
Mansehra
Pakistan

Part 1

Integrated Methods for Pest Control

1

Integrated Pest Management and Spatial Structure

Ernesto A. B. Lima[1], Wesley A. C. Godoy[2] and Cláudia P. Ferreira[3]
[1]*Programa de Pós graduação em Modelagem Computacional, LNCC*
[2]*Depto de Entomologia e Acarologia, ESALQ, USP*
[3]*Depto de Bioestatistica, IBB, UNESP*
Brazil

1. Introduction

During long time pest control was associated only to insecticides. The formulations were produced as an attempt of improving the insect control. However, some undesirable effects emerged in this time, mainly the toxin action and resistance. Insecticides are substances produced from chemical or biological products to control insect pests. The most common mode of action for insecticides is to kill insects by blocking physiological or biochemical processes (Ware & Whitacre, 2004). Usually, insecticides act on the nervous system, resulting in high efficacy and rapid responses in pest-control programs. Insecticides can be classified as physical, protoplasmic, metabolic inhibitors, neurotoxins, and hormone agonists (Matsumura, 1985). Mineral oil is an example of a physical insecticide, and heavy metals are protoplasmic insecticides (Amiri-Besheli, 2008; Gallo et al, 2002).

Some examples of metabolic inhibitors are the inhibitors of multi-function oxidases, carbohydrate and amino-acid metabolism inhibitors, and chitin-synthesis inhibitors (Krieger, 2001). The neurotoxins act through acetylcholinesterase, the neurotoxin that affects ion permeability, intervening in the nerve receptors of insects (Haynes, 1988), killing the arthropod by disrupting the membrane integrity (Gill et al, 1992).

The main groups of insecticides can be studied using the following classification: neurotoxins, insect growth regulators, cellular respiration inhibitors, and others. Of the neurotoxins, the organophosphates and carbamates act on synaptic transmission, accumulating acetylcholine molecules in the synapse, which in insects can produce a cholinergic syndrome characterized by nerve hyperexcitation (Costa et al, 2008; Thacker, 2002).

The acetylcholine agonists nicotines, neonicotinoids (the newest group of synthetic insecticides) and spinosines connect to the nicotine receptors of acetylcholine located in the pre-synaptic neuron (Thacker, 2002). In this case, the nerve impulses are continuously transmitted, also resulting in nerve hyper-excitation in the insect (Thacker, 2002). The acetylcholine antagonists avermectin and milbemycin block the nerve stimulus, immobilizing the insect. Cyclodienes and phenyl-pyrazoles act differently from avermectin and milbemycin, killing insects by inducing hyperexcitability (Thacker, 2002). DDT (dichlorodiphenyltrichloroethane) and pyrethroids are sodium-channel modulators, acting on sodium channels of nerve cells in insects (Thacker, 2002). Action potentials can be repetitive,

also killing insects by inducing hyperexcitability. On the other hand, the oxadiazines are sodium-channel blockers, reducing the ascendant phase of the action potential (Gallo et al, 2002).

Insect growth regulators such as chitin synthesis inhibitors are juvenile hormone agonists that slow development, producing an additional instar or nymph and preventing the insect from reaching the adult stage (Thacker, 2002). The juvenile hormone antagonists produce the opposite effect, forcing the insect to pass to the next life stage too early. Other inhibitors include cellular respiration inhibitors, which act by inhibiting respiratory-chain enzymes with consequent depression of respiratory movements and reduction of oxygen consumption; and adenosine triphosphate inhibitors, which inhibit oxidative phosphorylation (Hien et al, 2003).

The use of pesticides has been informally reported since 1000 B.C., but insect chemical control began in World War II, when the concept of insect control became established, opening a new era of synthetic organic insecticides, of which DDT was the first to be applied (Ware & Whitacre, 2004). DDT belongs to the organochlorines, insecticides containing carbon, hydrogen and chlorine, and is probably the most famous pesticide of the 20th Century. It is still used for malaria control in developing countries (Ware & Whitacre, 2004). Another notorious insecticide is BHC, which acts similarly to DDT but more rapidly.

The mid-20th Century saw the development of many pesticides and organophosphates, insecticides based on phosphorus; their development was also hastened during World War II, when they were tested to replace nicotine, mainly in Germany (Thacker, 2002). Because of the high toxicity of this pesticide it has been not recommended since 1990. Among the most common organophosphorus insecticides are malathion, monocrotophos, dicrotophos and methamidophos (Gullan & Cranston, 2005). Organosulfurs are less toxic pesticides that have been employed as acaricides. They differ from DDT in having sulfur in place of carbon. Carbamates, another class of defensives, are derivatives of carbamic acid; the first available member of this class was carbaryl, first marketed in 1956. The carbamates show low oral and dermal toxicity to mammals, and a broad spectrum of insect species are sensitive to the product (Thacker, 2002).

After this era, another class of pesticides was proposed, the pyrethroids. Pyrethroids are obtained from pyrethrum, a natural compound extracted from dried flowers of *Chrysanthemum cinerariifolium* and *C. coccineum* (Gullan & Cranston, 2005) and are much less toxic than organophosphates and carbamates. Their relatively low toxicity is associated with nonpersistent sodium-channel modulators (Dent, 2000). More recently, the synthetic neonicotinoids have been developed; these are analogues of the natural insecticide nicotine (Ware & Whitacre, 2004). They are nicotinic acetylcholine receptor agonists, with a broad spectrum and rapid action to replace the organophosphate and carbamate applications. Biological insecticides have been developed in order to avoid the application of chemical toxins to crops. Perhaps the most important idea was to use the endotoxins produced by *Bacillus thuringiensis* (BT), which acts by disintegrating epithelial cells of the mesentery (Gill et al, 1992). Some plants have been genetically modified to express BT toxins.

In spite of the great variety of pesticide formulations that act in different ways to control pests, the resistance of insects to insecticides has been reported frequently and over a long period. The definition of resistance proposed by the Insecticide Resistance Action Committee (IRAC) is "the selection of a heritable characteristic in an insect population that results in the repeated failure of an insecticide product to provide the intended level of control when used as recommended" (IRAC, 2010). Resistance to insecticides has been reported since 1914, initially for DDT, but has extended to new insecticide classes including cyclodienes, carbamates,

formamidines, organophosphates, pyrethroids and even *B. thuringiensis* (IRAC, 2010; Thacker, 2002). For this reason, the 1940s saw the beginning of more systematic investigations of the indiscriminate use of insecticides.

The toxicity of insecticides has been regularly discussed in the context of environmental contamination and human health (Wilson & Tisdell, 2001). However, there is no doubt of the importance of discussing their effects on animals, plants and the environment, particularly considering global warming. For example, of the organochlorines, the cyclodienes, which also appeared during World War II, have a different mode of action from DDT, and their toxicity increases with increasing temperature (Ware & Whitacre, 2004). In spite of the recent discussions of global warming with respect to diseases and insect dynamics (Lima et al, 2009), no systematic discussion has analyzed the possible effects of global warming on the toxicity of pesticides, except for a few isolated experiments (Gordon, 2003). This subject deserves special attention, taking into account that the earth's surface is predicted to warm by approximately 1.5 °C to 6 °C by the year 2100 (Kiritani, 2006).

Considering the risks of resistance, toxicity, and increase of toxicity associated with rising temperatures, the development of new pest-control methods should be encouraged to minimize the undesirable effects of pesticides. The use of traditional pesticides should be controlled to avoid the problems previously mentioned (Thompson & Head, 2001). The challenge is to identify and develop crop protection systems that integrate many measures to reduce and maintain a particular pest population at an acceptable level of economic damage (Radcliffe et al, 2009). One of the first articles involving the principles of Integrated Pest Management (IPM) was written in 1976 by Ray et al. (Smith & Calvert, 1976). This technique has been implemented for pest control, with acceptable results for different agricultural systems, and also to control disease vectors (Lima et al, 2009). The goal of IPM principles is to improve crop yield with minimum cost, taking into consideration the ecological and sociological constraints imposed by the particular agroecosystem under study and the long-term preservation of the environment. To achieve this, IPM techniques use a variety of approaches: first, to increase the knowledge of the insect pest and its relationship to the crop and factors affecting their interaction; second, to develop several techniques such as biological pest controls, farming practices, mechanical, and physical controls to reduce pesticide application; third, to improve methods of collecting and interpreting biological, meteorological, and crop production data; fourth, to build models of the crop production, pest dynamics, and management tactics integrated with an economic analysis to optimize crop yield; and finally, to conduct laboratory and field experiments to test these models (Smith & Calvert, 1976).

Therefore, an important strategy in any pest-control program is to determine the essential components of IPM in order to monitor the pest's status in the system. Monitoring of a pest's abundance in time and space is a powerful tool that provides information needed to decide on the best time to effectively implement control actions, by using insecticide applications and/or combining these methods with biological control strategies (Lima et al, 2009). The establishment of plans for sampling populations is an essential part of IPM programs, since it provides support for decision-making based on the pest density, forecast, and economic threshold (Beinns et al, 1992; Spencer et al, 2009). Ecological modeling is one of the most important initial components in IPM programs (Lima et al, 2009). By using models, it is possible to understand better the processes that govern biological systems of pest insects, because they describe very well the complexity involved in the population dynamics of

species from simple assumptions incorporated in the theoretical formalism (Faria & Godoy, 2001; Serra et al, 2007).
In the 1980s, IPM principles began to be used to control insect populations in urban sites, such as schools, parks, hospitals, and nursing homes; and following these ideas, in the 1990s mathematical models began to be constructed to analyze and discuss IPM methods in a more qualitative and quantitative way (Lima et al, 2009; Tang et al, 2005; Tang & Cheke, 2008). In particular, Tang and coworkers demonstrated a stable periodic solution in a prey-dependent consumption model with fixed impulsive effects, and gave an analytical expression for the period of this periodic solution. This period plays an important role in pest control, because it can be used to alter an IPM strategy with unfixed times for interventions, to one with periodic interventions, thus minimizing the cost of pest monitoring (Tang et al, 2005).
Recently, an extension of the Nicholson & Bailey model was proposed by Tang & Cheke, including the Integrated Pest Management strategies, in order to consider the economic threshold in the formulation. The study showed that the host level can be maintained below the economic threshold (ET), avoiding reaching the economic injury level (EIL). The study by Tang & Cheke (2008) also showed that high initial densities of parasitoids and high parasitoid inter-generational survival may lead to more frequent host outbreaks and, therefore, greater economic damage (Tang & Cheke, 2008). Lima and coworkers, using the formulation of a coupled map lattice, added spatial structure to this model, and showed that it can significantly alter the economic threshold-level values, which is an important aspect to consider in the success of the IPM technique (Lima et al, 2009).
In conclusion, the theory of pest control is closely associated with the basic principles of ecological theory, since its emphasis involves essentially the use of biological control strategies, which emerge from the classical theory of predator-prey relationships, with special application to insects (Hassell, 1978; Hochberg & Ives, 2000). In this chapter, we intend to show how theoretical ecology and pest-management strategies can be combined to facilitate the comprehension of important ecological aspects of a pest population, which directly influence its dynamics as well as the dynamics of its natural enemies. Theoretical models can address the relevance of intrinsic and extrinsic factors that affect the spatial and temporal dynamics of a biological system, and can also be useful to investigate different scenarios about its control.

2. Mathematical model

In this section, a non-spatial and also a spatial model will be developed to analyze preliminarily the contribution of several factors that can contribute to the effectiveness of the IPM methodology.

2.1 Population dynamics without MIP

Let us suppose a hypothetical pest of a crop that has a natural enemy, an insect that is a parasitoid, and also a predator of this pest. As a example, we can cite *D. citri* as a pest of the orange crop, and *T. radiata* as its natural enemy. In this case, the natural enemy can attack different stages of the pest's life cycle, acting as a good candidate for biological control (Fauvergue & Quilici, 1991). All mathematical models must be constructed based on the life cycles of the populations that are relevant for the process under study. Also, the complexity arises from the standpoint of mathematics and computing leads us to make simplifications in modeling the biological problem. Therefore, bearing in mind these two insect species and the host-parasitoid-prey-predator interaction, let us divide the pest's life

cycle into four compartments: egg (O), two nymphs (N_1, N_2), and the adult female (F); which represents the number or density of individuals in a specific development stage at time t. The variables N_1 and N_2 represent the number of individuals undergoing predation and parasitism, respectively. With respect to the natural enemy, we will consider two development stages, one the juveniles (J) and the other the adult female (P). Also, we adopted for the interaction between the populations, the Type II function response, where consumption and parasitism rise asymptotically to saturation. Thus, the following system of differential equations describes the temporal evolution of the individuals in each compartment:

$$\begin{cases} \frac{dO}{dt} &= \eta F\left(1-\frac{O}{K}\right)-(\sigma_o+\mu_o)O, \\ \frac{dN_1}{dt} &= \sigma_o O-(\sigma_{n_1}+\mu_{n_1})N_1-\frac{\gamma N_1 P}{1+\phi N_1}, \\ \frac{dN_2}{dt} &= \sigma_{n_1}N_1-(\sigma_{n_2}+\mu_{n_2})N_2-\frac{\psi_t \alpha N_2 P}{1+\beta N_2}, \\ \frac{dF}{dt} &= \psi_c \sigma_{n_2}N_2-\mu_f F, \\ \frac{dJ}{dt} &= \theta\frac{\psi_t \alpha N_2 P}{1+\beta N_2}-(\sigma_j+\mu_j)J, \\ \frac{dP}{dt} &= \sigma_j J-\mu_p P\left(1-\omega\frac{\gamma N_1}{1+\phi N_1}\right). \end{cases} \tag{1}$$

In the first equation, we assume a logistic population growth for the number of eggs, where η is the per capita oviposition rate and K is the carrying capacity, since oviposition occurs in new shoots, as is usual in a large number of crops. The number of eggs decreases due to the natural per capita mortality rate μ_o, and by the per capita eclosion rate σ_o. In the second equation, the nymph population N_1 increases by the eclosion of eggs and decreases by the per capita natural mortality, μ_{n_1}, and due to transition to the N_2 stage at the per capita rate σ_{n_1}, and predation, where γ is the per capita predation rate and ϕ is related to the pray handling time. In the third equation, the population N_2 increases by the transformation of N_1 into N_2 and decreases by the natural per capita mortality rate μ_{n_2}, transition to the adult phase at a per capita rate σ_{n_2}, and due to parasitism, where ψ_t is the sex ratio, α is the per capita parasitism rate and β is related to the host handling time. Finally, adult females increase as $\psi_c\sigma_{n_2}$, where ψ_c is the sex ratio, and decrease by the natural per capita mortality rate μ_f.
For the natural enemy, we assume that it is a specialist parasitoid, and therefore juveniles increase when the female emerges from a host, where θ is the mean number of juveniles; and decrease by the natural per capita mortality rate μ_j and by transition to the adult stage at the per capita rate σ_j. The adult population increases by the transformation of juveniles to adults and decreases by the per capita mortality rate μ_p. Furthermore, we will assume that the enemy's natural mortality rate decreases by $\omega < 1$ because experimental results show that predation, in general, increases the survival of the predator.

2.2 Adding IPM to the mathematical model

The size of the insect population is affected by extrinsic factors such as the amount of available food and the weather. Therefore, the spatial-temporal pattern for the number of individuals observed in the field shows periodic oscillations with different amplitudes and spatial heterogeneity. An insect becomes a pest when it exceeds some threshold related to its population size, and begins to cause economic injury to the producer, and also to impact the local or global economy. Integrated pest management programs use current, comprehensive information on the life cycles of pests and their interaction with the environment. This

technique relies on monitoring and identifying pests and their natural enemies, setting an action threshold, prevention methods, and control. It is an interesting alternative to the use of pesticides that, besides leading to pesticide-resistance problems, contaminate food, soil and water and remove a pest's natural predators and other non-target species. In fact, the IPM concept incorporates an array of management tactics including biological pest control, farming practices, and mechanical, physical and chemical controls. In this study, we are dealing with two of them, pesticide spraying and parasitoid release.

In order to add IPM strategies to the system described in (1), we must remember that in IPM programs, both pesticide spraying and natural enemy release occur when the pray population density reaches the economic threshold, ET, in order to maintain the pray density below the economic injury level, EIL (see Fig. 1). Accurate determination of these two thresholds require knowledge of the pest, plant health problems, and what constitutes unacceptable pest damage. Thus, each insect-ecosystem has its specific thresholds. To incorporate these two processes, pesticide spraying and parasitoid release, into an IPM program, the system (1) should be written as

$$\begin{cases} \left.\begin{aligned} \tfrac{dO}{dt} &= \eta F\left(1-\tfrac{O}{K}\right)-(\sigma_o+\mu_o)O,\\ \tfrac{dN_1}{dt} &= \sigma_o O-(\sigma_{n_1}+\mu_{n_1})N_1-\tfrac{\gamma N_1 P}{1+\phi N_1},\\ \tfrac{dN_2}{dt} &= \sigma_{n_1}N_1-(\sigma_{n_2}+\mu_{n_2})N_2-\tfrac{\psi_t\alpha N_2 P}{1+\beta N_2},\\ \tfrac{dF}{dt} &= \psi_c\sigma_{n_2}N_2-\mu_f F,\\ \tfrac{dJ}{dt} &= \theta\tfrac{\psi_t\alpha N_2 P}{1+\beta N_2}-(\sigma_j+\mu_j)J,\\ \tfrac{dP}{dt} &= \sigma_j J-\mu_p P\left(1-\omega\tfrac{\gamma N_1}{1+\phi N_1}\right), \end{aligned}\right\} \text{if } F<ET,\\ \left.\begin{aligned} O(t_0^+) &= (1-\delta_c)O(t_0),\\ N_1(t_0^+) &= (1-\delta_c)N_1(t_0),\\ N_2(t^+) &= (1-\delta_c)N_2(t_0),\\ F(t_0^+) &= (1-\delta_c)F(t_0),\\ J(t_0^+) &= (1-\delta_c)J(t_0),\\ P(t_0^+) &= (1-\delta_c)P(t_0)+\tau, \end{aligned}\right\} \text{if } F\geq ET, \end{cases} \tag{2}$$

where

$$\delta_c = 1-\frac{ET}{F}, \tag{3}$$

is the instantaneous per capita mortality rate in response to the pesticide applied at $t=t_0$, τ is the number of parasitoids released at this time, and $O(t_0^+)$, $N_1(t_0^+)$, $N_2(t_0^+)$, $F(t_0^+)$, $J(t_0^+)$ and $P(t_0^+)$ are the number of individuals in each class after pesticide application (Tang & Cheke, 2008). To simplify, we are assuming that control measures affect all pest and natural-enemy stages with the same intensity.

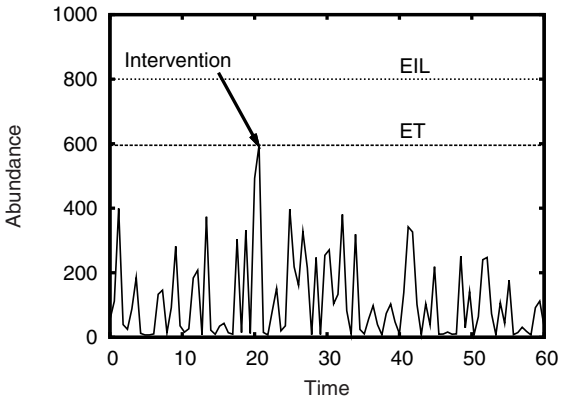


Fig. 1. Temporal evolution of the insect population, economic injury level (EIL) and economic threshold (ET).

2.3 Adding spatial structure to the mathematical model

To consider the spatial structure, we use the formulation of coupled lattice models. The bidimensional lattice with 4×4 sites, represents the crop plots (see Fig. 2). Each plot consists of 45×45 sites, each of which represents a specific tree that serves as a source of food for the pest, e.g., an orange tree. Dispersal occurs between adjacent sites, considering the Moore neighborhood of one radius. We implemented an asynchronous lattice update and a fixed boundary condition. At each site, the pest and natural enemy populations are arranged in such a way that each equation of the system (2) receives an index i that refers to a grid cell within the lattice. In each grid cell, the system is solved using the Runge-Kutta 4th-order method.

At each simulation time step, which corresponds to one day, the dynamics consist of three phases: population dynamics (reproduction-parasitism-predation phase), dispersal phase, and population control. The results are shown using $\nu_d = 0.85$ and $\nu_t = 0.6$ for the dispersal of the pest and its natural enemy, respectively. Therefore, at each time step, the fraction of the pest and the natural enemy populations that undergo migration are $\nu_d F/8$ and $\nu_t P/8$. The other parameters are $\eta = 9.880, \sigma_0 = 0.1422, \mu_0 = 0.1060, \sigma_{n_1} = 0.1031, \mu_{n_1} = 0.2029, \sigma_{n_2} = 0.08292, \mu_{n_2} = \mu_j = 0.01892, \mu_f = 0.01976, \sigma_j = 0.05882, \mu_p = 0.02941$ all in day^{-1}, $\beta = 0.6, \phi = 0.6$ in day, $\theta = 0.7, \alpha = 0.1, \omega = 0.2, \gamma = 0.2, \psi_c = 0.5, \psi_t = 0.6429$ and $K = 7500$. Again, the parameter values were chosen bearing in mind the *D. citri* and *T. radiata* interaction system (Liu & Tsai, 2000; Pluke et al, 2008). Indeed, the same qualitative results are obtained for other set parameters, and can be discussed in another ecological system in context. For each specific system of pray-natural enemy, these parameters must be estimated in laboratory and field experiments.

We started by randomly choosing 20 sites (in the order of 1% of the total lattice) to quantify the number of female adult insects, F, above the economic threshold, ET. Therefore, if more than two sites (in the order of 20% of the total analyzed) have $F > ET$, we applied insecticide followed by release of the parasitoid. We chose to apply the control techniques in the same way as is usual to control the Citrus Variegated Chlorosis (CVC), disease caused by a xylem-inhibiting bacterium *Xylella fastidiosa* and transmitted by 11 species of sharpshooter leafhoppers, because for the *D. citri* and *T. radiata* interaction system, the control strategy is

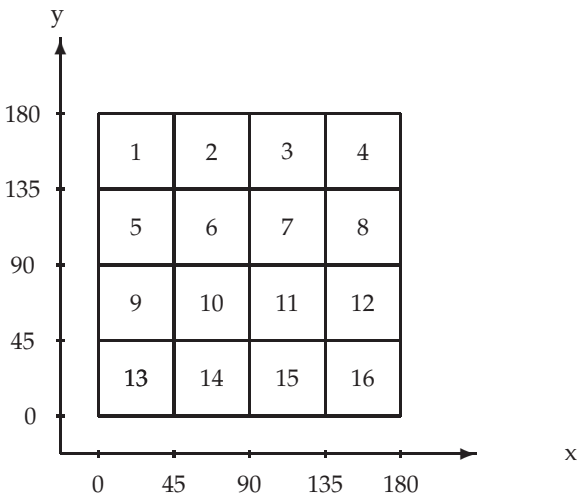


Fig. 2. Lattice used in the simulations. Each plot consists of 45×45 sites that represent the source of food for the pest. In each site, the dynamic system (2) is solved and dispersal occurs between adjacent sites.

still being developed (remember that *D. citri* is a vector of *Liberibacter sp.*, the causal agent of huanglogbing (HLB), and in these circumstances the idea of an ET is difficult to implement). In a real situation, only a proportion of the crops are monitored periodically, and the control is applied or not based on the analysis of these random samples. We also assume that the proportion of individuals that die by pesticide application is proportional to the highest value of female pests obtained in this analysis. Also, the control techniques are applied in the same way to all crops.

3. Results and discussion

In this section, the model will be analyzed to gain insight into its dynamic features.

3.1 Model without and with IPM and non-spatial structure

Let us begin by analyzing the non-spatial model without IPM strategy. Equilibria for the system (1) are found by setting the right half of each equation equal to zero. It can be seen that the model accepts three equilibria:

- *Trivial equilibrium* given by $E_0 = (0, 0, 0, 0, 0, 0)$, corresponding to the state where the populations of the pest and the natural enemy are absent;
- *Pest persistence and natural enemy exclusion* given by $E_1 = (O^*, N_1^*, N_2^*, F^*, 0, 0)$, corresponding to a state where the pest population persists while the natural enemy is absent;
- *Coexistence of the two populations* given by $E_2 = (O^\star, N_1^\star, N_2^\star, F^\star, J^\star, P^\star)$, corresponding to the state where both populations are present.

The stability analysis is given by the eigenvalues of the characteristic equation $\Delta(\lambda) = det(J^* - \lambda I) = 0$, evaluated at each equilibrium point, where J^* is the Jacobian matrix (linearization of the system dynamics) and I is the identity matrix. After some algebraic

manipulation, we are able to determine threshold values that divide the solution space. Thus, E_0 is locally asymptotically stable if $R_{c_1} < 1$, and unstable if $R_{c_1} > 1$, where

$$R_{c_1} = \frac{\eta\sigma_o\sigma_{n_1}\sigma_{n_2}\psi_c}{(\sigma_o+\mu_o)(\sigma_{n_1}+\mu_{n_1})(\sigma_{n_2}+\mu_{n_2})\mu_f}. \tag{4}$$

In demographic terms, R_{c_1} is the *basic reproductive number* of the pest population (equivalent to the basic reproductive number in the epidemiological context). Interestingly, R_{c_1} does not depend on the interaction between the pest and its natural enemy. When $R_{c_1} > 1$, the pest population is able to maintain itself in the field, and the equilibrium E_1 emerges in the feasible region. The stable state of E_1 is thus obtained when $R_{c_2} < 1$, where

$$R_{c_2} = \frac{(KA)^2(l\mu_p\omega\gamma\beta + \psi_t\sigma_j\theta\alpha\phi) + KAB(\sigma_{n_1}\psi_t\sigma_j\theta\alpha + fl\omega\gamma\mu_p)}{\mu_p(2B^2f\sigma_{n_1} + (KA)^2l\beta\phi + KAB(\sigma_{n_1}l\beta + fl\phi))}, \tag{5}$$

and

$$\begin{cases} f &= \sigma_{n_2} + \mu_{n_2}, \\ l &= \sigma_j + \mu_j, \\ A &= \eta\psi_c\sigma_{n_2}\sigma_{n_1}\sigma_o - (\sigma_o+\mu_o)(\sigma_{n_1}+\mu_{n_1})(\sigma_{n_2}+\mu_{n_2})\mu_f, \\ B &= \psi_c\sigma_{n_2}\eta(\sigma_{n_1}+\mu_{n_1}). \end{cases} \tag{6}$$

Therefore, E_1 is locally asymptotically stable when $R_{c_2} < 1$ and unstable when $R_{c_2} > 1$. The equilibrium E_2 was analyzed numerically. It seems that for a suitable parameter value, a periodic solution around the equilibrium E_2 can appear. In this case, the dynamic features of the system depend on the interaction between the two populations that comprise the biological system. Because we are interested in discussing a pest-control technique, the results were obtained for parameter values that give $R_{c_1} > 1$ and $R_{c_2} > 1$.

Fig. 3 shows the temporal evolution of the pest and natural-enemy populations, for the non-spatial and non-ET model (equation (1)) using the parameter set described above, in this case, $R_{c_1} = 39.3$ and $R_{c_2} = 2.0$. The temporal pattern obtained for the two populations exhibits periodic oscillations with a maximum amplitude of 18000 individuals for the pest, and a period of 2 years. The increase of the pest population is followed by the increase of the parasitoid-predator population, with a maximum amplitude of 4000 individuals.

Fig. 4 shows the temporal dynamics of the system when an IPM program is in progress. The economic injury level was defined as $EIL = 150$, and the system dynamics was managed to allow the pest density to fall below the EIL level. In order to achieve our goal, we must consider the economic threshold as $ET = 30$. On the order of $\tau = 20$ parasitoid adults are released. As a result, Fig. 4, shows that population coexistence is maintained, but the amplitudes of the pest and natural enemy oscillations decrease respectively to 1200 and 400 individuals. For this parameter set, both the optimum times of the applications, Δ_t, and the percentage of the pests that needs to be eliminated with pesticides can be estimated (plotting δ_c given by equation (3) as a function of time) resulting in a periodic application of pesticide at intervals of 21 days, and the pesticide should kill approximately 6.2% of the population. Also, the periods of oscillation for both populations decrease to less than one year, and the lag time observed between the temporal dynamics of the two populations decreases.

To analyze the influence of the ET on the determination of Δ_t, in Fig. 5(a) we ran several simulations, varying ET and estimating Δ_t necessary to maintain the pest population below the EIL. The other parameters are the same as in Fig. 4. The results are shown for two different values of τ, which measure the number of parasitoids released. The solid and dotted

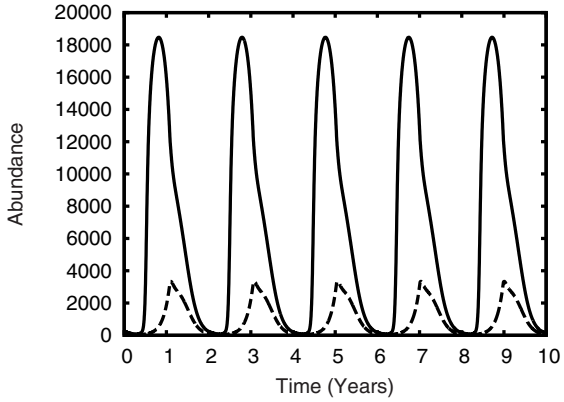


Fig. 3. Temporal evolution of the total pray (solid line) and natural enemy (dashed line) population for the non-spatial model without IPM strategy.

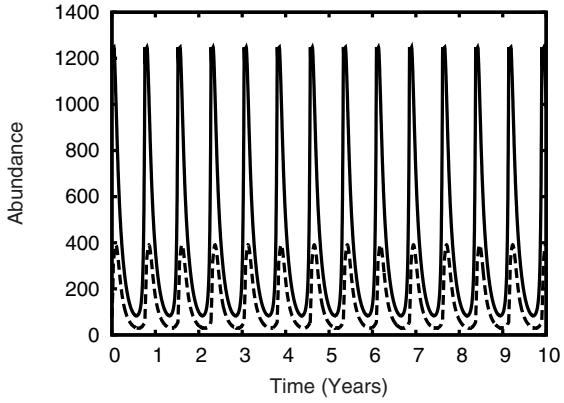


Fig. 4. Temporal evolution of the total pest (solid line) and natural enemy (dashed line) populations for the non-spatial model with IPM strategy

lines divide the $(\Delta_t - ET)$ parameter space into two regions; below the curve, the EIL is not exceeded; and above the curve, the pest population is greater than the EIL. Also, the qualitative behavior of Δ_t versus ET seems to be a rational function such as $\Delta_t = a_0/(a_1 + a_2 ET)$. Moreover, the number of times that the IPM technique must be applied, N_p, increases almost linearly with ET, $N_p = b_1 + b_2 ET$, with a linear slope of 4.6 for $\tau = 20$ and 2.2 for $\tau = 30$ (Fig. 5(b)). In brief, the values of a_0, a_1, a_2, b_1 and b_2 depend on the parameters set, but the qualitative behavior of the curves does not change. Finally, an increase τ leading to the increase of Δ_t and decrease of N_p, indicates a range of possible strategies that may be associated with an economic cost to the producer.

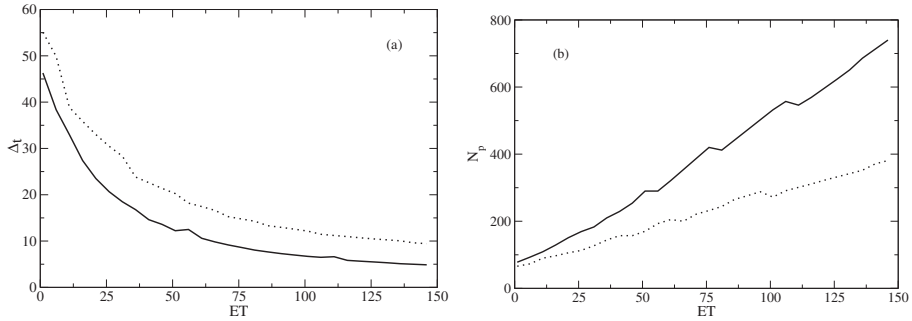


Fig. 5. In (a) interval between pesticide application, Δ_t, versus economic threshold, ET. The lines divide the $(\Delta_t - ET)$ parameter space into two regions, and are obtained for different τ values. Below each line, the IMP technique succeeds, and above it the technique fails. In (b) the number of applications in 15 years versus ET. The solid line corresponds to $\tau = 20$ and the dotted line to $\tau = 30$.

3.2 Spatial model with IPM

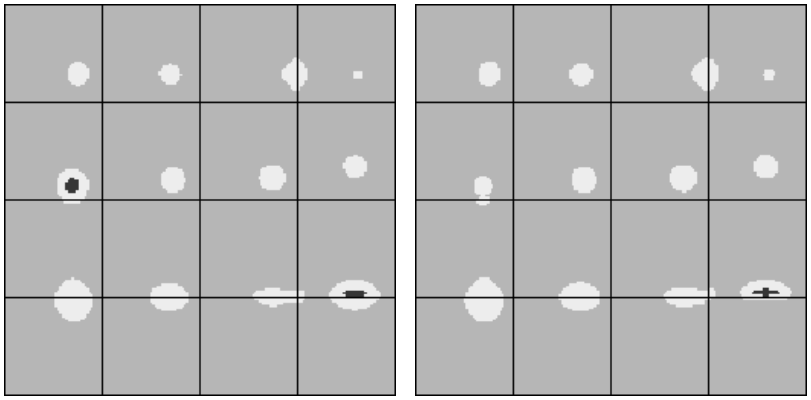
The simulation starts with a random selection of a site in each crop, to be occupied by a pest and a natural enemy individual, and all other lattice sites are empty (simulating an initial invasion-colonization of the crop, in which a small number of individuals arrived first). Therefore, the system dynamics (reproduction-parasitism-predation phase, dispersal phase, and population control) evolves and a snapshot of the lattice configuration in different time steps can be analyzed.

Fig. 6 shows the spatial distribution for the pest adult females for $\tau = 20$ using the parameter set described in Fig. 4. Different levels of shading represent different numbers of pests, respectively, $F < ET$ (gray), $ET \leq F < EIL$ (white) and $F \geq EIL$ (black). Because we are considering a homogeneous diffusion (with no preferential direction), the observed pattern is a symmetrical wave front started at each initial occupied site. Interestingly, this leads to a larger number of pests at the border of the crop, which is observed in the field.

Fig. 6 shows two snapshots of the lattice configuration at different time steps. Following the crop numeration shown in Fig. 2, we conclude that in crops 5 and 16, IPM control was applied. However, control efforts depend on the estimate of the number of adult female pests, F, which also depends on the crop-site sampling. As a result, we can see a higher efficacy of IPM for crop 16 compared with crop 5. Moreover, we can see a reinfestation of crop 16 as a result of the migration of the pest population from crop 12, where IPM control was not applied.

Fig. 7 shows the influence of the ET on the determination of Δ_t for the spatial model. In order to discuss the importance of the spatial structure for the IPM technique, the results obtained for the non-spatial model are also added. We can see that the non-spatial model also overestimates the time interval for the IMP application, leading to failure of the technique. For each value of ET, we are able to calculate the percentage of the lattice with $F \geq EIL$ that gives a measure of the economic damage to the producer. For $ET = 30, 60$ and 90, we obtain, respectively, 0.352%, 0.512% and 0.921% of the lattice site with F above EIL. The results plotted for the spatial model are the mean values of 47 simulations for each value of ET.

In a recent study, Lima and coworkers showed that the ET level should be lower than the value suggested by non-spatial models, to assure that pest density remains below the EIL



(a) $t = 700$ (b) $t = 701$

Fig. 6. Different levels of shading represent different numbers of the pest, respectively, $F < ET$ (gray), $ET \leq F < EIL$ (white) and $F \geq EIL$ (black). In (a) snapshot of the lattice configuration at time $t = 700$ and in (b) for time $t = 701$.

level (Lima et al, 2009). Looking at Fig. 7, we can see that the difference in the Δ_t values obtained for a non-spatial and a spatial model is greater for small values of ET. Certainly, these results show that the spatial structure affects the ET level, and consequently also the interval between applications of IPM, and seems to be the main reason for the failure of the technique.

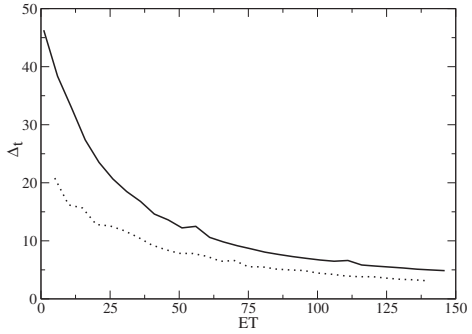


Fig. 7. Interval between pesticide application, Δ_t, versus economic threshold, ET. The solid line corresponds to the non-spatial model, and the dotted line to the spatial model.

4. Conclusion

Ecological modeling is an important tool for systematic study of the use of the IPM technique to control insect populations. Different scenarios can be planned and tested prior to implementation, making experimental designs more efficient and saving time and money. Of course, every mathematical model is a caricature of the real biological system, and

the knowledge of the insect pest and its relationship to the crop and factors affecting the interaction between them determines the degree of accuracy of a model's predictions.
In this contribution, we discuss the interaction between a hypothetical crop pest that has a natural enemy, an insect that is a parasitoid, and also a predator of this pest. Using this host-parasitoid-prey-predator system, we discuss the use of pesticide spraying and parasitoid release to control the pest population. For each parameter set of the model, we were able to predict the time interval between successive applications of the IPM technique, and also the number of applications as a function of the economic threshold. As shown in Fig. 5, these two factors are important in determining the success or failure of the IPM methodology.
Finally, the spatial model shows how the spatial structure can affect the effectiveness of the technique. The non-spatial model always overestimates the interval between IPM applications, and also the number of applications (Fig. 7). As a rule, for the spatial model, increasing the economic threshold makes pest control more difficult, leading to an increase in the economic damage. As a future study, it will be interesting to add the influence of temperature on the entomological parameters of the insects, and also the temporal and spatial dynamics of the target crop, to analyze how these phenomena affect the IPM methodology.

5. References

Amiri-Besheli, B. (2008). Efficacy of *Bacillus thuringgiensis*, mineral oil, insecticidal emulsion and insecticidal gel against *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae), *Plant Protection Science* Vol. 44: 68-73.

Beinns, M. R., Nyrop, J. P. (1992) Sampling insect populations for the purpose of IPM decision making, *Ann Rev Entomol* Vol. 37: 427-453.

Costa, L. G., Giordano, G., Guizzetti, M., Vitalone, A.(2008). Neurotoxicity of pesticides: a brief review, *Frontiers of Bioscience* Vol. 13: 1240-1249.

Dent, D. (2000). *Insect Pest Management*, Cabi Bioscience, UK Centre, Askot, UK.

Faria, L. D. B. & Godoy, W. A. C. (2001). Prey choice by facultative predator larvae of *Chrysomya albiceps* (Diptera: Calliphoridae), *Memórias do Instituto Oswaldo Cruz* Vol. 96: 875-878.

Fauvergue, X. & Quilici, S (1991). Studies on the biology of *Tamarixia radiata* (Waterston, 1922) (Hymenoptera: Eulophidae), primary ectoparasitoid of *D. citri* Kuwayama (Hemiptera, Psyllidae), Asian vector of citrus greening disease, *Fruits* Vol. 46: 179-183.

Gallo, D., Nakano, O., Silveira Neto, S., Carvalho, R. P. L., Baptista, G. C., Berti Filho, E., Parra, J. R. P., Zucchi, R. A., Alves, S. B., Vendramim, J. D., Marchini, L. C., Lopes, J. R. S. & Omoto, C. (2002). *Entomologia Agrícola*, FEALQ, Piracicaba.

Gill, S. S., Cowle, E. A. & Pietrantonio, P. V. (1992). The mode of action of Bacillus thuringiensis endotoxins, *Annual Review of Entomology* Vol. 37: 615-634.

Gordon, C. (2003). Role of environmental stress in the physiological response to chemical toxicants, *Environmental Research* Vol. 92:1-7.

Gullan, P. J. & Cranston, P. S. (2005). *The insects, an outline of entomology*, Blackwell Publishing, Malden, USA.

Haynes, K. F. (1988). Sublethal effects of neurotoxic insecticides on insect behavior, *Annual Review of Entomology* Vol. 33: 149-168.

Hassell, M. P. (1978). *The dynamics of arthropod predator-prey systems*, Princeton University Press, Princeton, N.J.

Hien, P. P., Gortnizka, H., Kraemer, R. (2003). Rotenone - potential and prospect for sustainable agriculture, *Omonrice* Vol. 11: 83-92.

Hochberg M.E. & Ives A.R. (2000). *Parasitoid Population Biology*, Princeton University Press, Princeton, N.J.

IRAC (2010). Prevention and Management of Insecticide Resistance in Vectors of Public Health Importance, Insecticide Resistance Action Committee (IRAC), Insecticide Resistance Action Committee (IRAC). URL:www.irac-online.org

Kiritani, K. (2006). Predicting impacts of global warming on population dynamics and distribution of arthropods in Japan, *Population Ecology* Vol. 48: 5-12.

Krieger, R. (2001). *Handbook of pesticide toxicology principles*, Academic Press, London, UK.

Lima, E.A.B; Ferreira, C.P.; Godoy, W.A.C. (2009). Ecological Modeling and Pest Population Management: a Possible and Necessary Connection in a Changing World, *Neotropical Entomology* Vol. 38(6): 699-707.

Liu, Y.H. & Tsai, J. H. (2000). Effects of temperature and life table parameters of the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Homoptera: Psyllidae), *Annals of applied biology* Vol. 137 (3): 201-206.

Matsumura, F. (1985). *Toxicology of Insecticides*, Plenum Press, New York, NY.

Pluke, R. W. H.; Qureshi, J. A.; Stansly, P. A. (2008). Citrus Flushing Patterns, *iaphorina citri* (Hemiptera: Psyllidae) Population and Parasitism by *Tamarixia radita* (Hymenoptera: Eulophidae) in Puerto Rico, *Florida Entomologist* Vol. 91 (1): 36-42.

Radcliffe, E.B.; Hutchison, W.D.; Cancelado, R.E. (2009). *Integrated Pest Managent: Concepts, Tactics, Strateies and Case Study*, Cambridge University Press.

Serra, H., Silva, I. C. R., Mancera, P. F. A., Faria, L. D. B., Von Zuben, C. J., Von Zuben, F. J., Reis, S. F., Godoy, W. A. C. (2007), *Ecological Entomology* Vol. 22: 686-695.

Smith, R.F.; Calvert, D.J. (1976). Health-Related Aspects of Integrated Pest Management, *Environmental Health Perspectives* Vol. 14: 185-191.

Spencer. J. L., Hibbard, B. E., Moeser, M., Onstad, D. (2009) Behaviour and ecology of the western corn rootworm *Diabrotica virgifera* LeConte, *Agric For Entomol* Vol. 11: 9-27.

Tang, S; Xiao, Y.; Chen, L.; Cheke, R.A. (2005). Integrated pest management models and their dynamical behaviour, *Bulletin of Mathematical Biology* Vol. 67: 115-135.

Tang S.; Cheke, R. A. (2008). Models for integrated pest control and their biological implications, *Mathematical Biosciences* Vol. 215: 115-125.

Thacker, J. R. M. (2002). *An introduction to arthropod pest control*, Cambridge University Press, Cambridge, UK.

Thompson, G. D., Head, G. (2001). Implications of regulating insect resistance management, *American Entomologist* Vol. 47: 6-10.

Ware, G. W., Whitacre, D. M. (2004). *The Pesticide Book*, Meister Media Worldwide, Willoughby, Ohio.

Wilson, C. & Tisdell, C. (2001). Why farmers continue to use pesticides despite environmental, health and sustainability costs, *Ecological Economics* Vol. 39: 449-462.

2

Ecosmart Biorational Insecticides: Alternative Insect Control Strategies

Hanem Fathy Khater
Medical and Veterinary Parasitology,
Faculty of Veterinary Medicine, Benha University,
Egypt

1. Introduction

Pest insects can damage agricultural crops, consume and/or damage harvested food, or transmit diseases to humans and animals. The past 30 years has witnessed a dramatic re-emergence of epidemic vector-borne diseases throughout much of the world (Atkinson, 2010). Prior to the development and commercial success of synthetic insecticides in the mid-1930s to 1950s, botanical insecticides were the foremost weapons against insect pests. The synthetic insecticides (organochlorines, organophosphates, carbamates and later the pyrethroids and neonicotinoids) are characterized by efficacy, speed of action, ease of use, and low cost. Accordingly, they drove many natural control methods, such as using of botanicals, predators, and parasitoids to near obscurity. Twenty years after synthetic insecticides were overzealously entrenched in 'modern' agricultural production; they induce widespread environmental contamination, toxicity to non-target organisms, development of resistance against insecticides, and negative effects on animal and human health (Pretty, 2009). Consequently, there is an urgent need to explore and utilize naturally occurring products for combating pests.

The terms "biorational pesticide" and "biopesticides" are gaining popularity in the current climate of environmental awareness and public concern. Both terms are derived from two words, "biological" and "rational", referring to pesticides that have limited or no adverse effects on the environment, non- target organisms including humans. Biorational insecticides include: biochemicals insecticides (botanicals, insect growth regulators, insect pheromones, photoinsecticides, and inorganics); biological insecticides, using of natural enemies such as parasitoids, predators, nematodes, and pathogens (virus, bacteria, fungi, or protozoa); and transgenic insecticides (genetically modified plants or organisms). Natural enemies play an important role in limiting potential pest populations and they are more likely to survive in case of application of ecofriendly biopesticides. Approaches to the biological control of insects include: conservation of existing natural enemies; introducing new natural enemies and establishing a permanent population (called "classical biological control"); and mass rearing and periodic release, either as a seasonal introduction of a small population of natural enemies, or a massive, "inundative" release. In developing countries, biopesticides offer unique and challenging opportunities for exploration and development of their own biorational insecticides. Nanotechnology has become one of the most promising

new technologies in the recent decade for protection against insect pests. Such technology will revolutionize agriculture including pest management in the foreseeable future.
Integrated pest management (IPM) is the use of all available means to maintain pest populations below levels that would cause economic loss while minimally impacting the environment. Several tactics could be utilized in IPM programs as chemical, cultural, physical, and biological control (Vreysen et al. 2007). The introduction of more effective biorational products through IPM programs will reduced rates of chemical pesticides and prevent, or at least delay the development of resistance in target pests to both chemical pesticides and biopesticide toxins. Flourishing of organically produced food in the developed world facilitates greater farmer acceptance of biopesticides as the sales of organically produced food are increasing at a significantly faster rate than sales of any other food commodity. Consequently, biorational insecticides will dominate the market of pesticides in the near future. Here, I am concerned about control of insects and arachnids (ticks and mites) of agricultural, medical, and veterinary importance and referred to them as insects or insect pests. The words "biorational" and "biopesticide" as well as "pesticides" and "insecticides" are used interchangeably throughout this chapter. Finally, I review current biorational insecticides and their mode of actions, uses, commercial products, and safety concerns.

2. Biochemical control

2.1 Botanical insecticides

The practice of using plant derivatives or botanical insecticides in agriculture dates back at least two millennia in ancient Egypt, India, China, and Greece. In Europe and North America, the documented use of botanicals extends back more than 150 years, dramatically predating discoveries of the major classes of synthetic chemical insecticides beginning in the 1940s.

2.1.1 Traditional botanical insecticides

2.1.1.1 Pyrethrum

Pyrethrum is one of the oldest and safest insecticides. The ground, dried flowers of *Tanacetum cinerariaefolium* (Asteraceae) were used in the early 19th century to control body lice during the Napoleonic Wars. Pyrethrum contains three esters of chrysanthemic acid and three esters of pyrethric acid. Among the six esters, those incorporating the alcohol pyrethrolone, namely pyrethrins I and II, are the most abundant and account for most of the insecticidal activity. Technical grade pyrethrum, the resin used in formulating commercial insecticides, typically contains from 20% to 25% pyrethrins (Casida & Quistad, 1995). Recently, Australia produces almost one-half of the world supply and produces a technical grade material comprising 50% pyrethrins by weight. Pyrethrins affect the insect on contact, creating disturbances in the nervous system which eventually result in convulsions and death. Pyrethrin acts on insects with phenomenal speed causing immediate paralysis, notably in flying insects, some of which are immobilized within 1 s. It blocks voltage-gated sodium channels in nerve axons. The mechanism of action of pyrethrins is qualitatively similar to that of DDT and many synthetic organochlorine insecticides. Pyrethrums are mixed with a synergist such as piperonyl butoxide (PBO) to increase insect mortality and to extend their shelf life. In purity, pyrethrins are moderately toxic to mammals, but technical

grade pyrethrum is considerably less toxic (Casida & Quistad, 1995). Major uses of pyrethrum are for structural pest control, in public health, and for treatment of animal premises. Pyrethrins have limited use outdoors as they are especially labile in the presence of the UV component of sunlight (Ware & Whitacre, 2004). Pyrethrum products represent 80% of the total market of global botanical insecticides (Isman, 2005) and are favored by organic growers because of their low mammalian toxicity and environmental non-persistence making it among the safest insecticides in use. For more information about pyrethrum, see Taylor (2001), Collins (2006), and Gilbert & Gill (2010).

2.1.1.2 Other traditional botanicals

A handful of other plant materials have seen limited commercial use as insecticides and their uses are in decline, such as sabadilla, a powder based on the ground seeds of the South American plant *Schoenocaulon officinale* ; Wood of the Caribbean tree *Ryania speciosa*; *Quassia amara,* a small tree from Brazil; woodchips and ground bark of this species have been used traditionally as an insecticide, as have plant parts from the related tree, *Ailanthus altissima*; rotenone, an isoflavonoid obtained from the roots or rhizomes of tropical legumes in the genera *Derris, Lonchocarpus,* and *Tephrosia*. Rotenone is used as insecticide and mainly a fish poison to paralyze fish, causing them to surface and be easily captured, but there is a growing concern about its safety and its relation to Parkinson's disease (Betarbet et al., 2000). For more niceties about traditional botanical insecticides**,** see Ware & whitecare (2004), Isman (2005, 2006, 2010), Isman & Akhtar (2007), Gilbert & Gill (2010), Kumar et al (2010), Dubey (2011), and Mehlorn (2011).

2.1.2 Newer botanical insecticide "Neem"

Neem (*Azadirachta indica* A. Juss: Meliaceae) is a large, evergreen, hardy tree, native to the Indian sub-continent and well known their as the' Botanical Marvel', It is an old and new insecticide. The Indians used neem, from prehistoric times, primarily against household and storage pests, and to some extent against pests related to field crops. In addition, they traditionally burn neem leaves in the evening to repel mosquitoes. It is effective against more than 500 species of insects and arthropods. Neem has attracted global attention recently due to its potential as a source of natural drugs and as environment-friendly pesticides, see Schmutterer (1995), Kumar (2002), Isman et al. (2011), and Mehlhorn (2011) for more fine points.

2.1.2.1 Chemical composition

Neem seeds are a rich storehouse of over 100 tetranortriterpenoids and diverse non-isoprenoids. The neem tree contains more than 100 different limonoids in its different tissues (Isman et al., 1996). Many of them are insect feed deterrents. The highly oxygenated azadirachtin ($C_{35}H_{44}O_{16}$), a nortriterpenoid belonging to the lemonoids, is the most biologically active constituent of neem. Azadirachtin has shown bactericidal, fungicidal, and insecticidal properties, including insect growth regulating qualities (Ware & Whitacre, 2004). It is systemic in nature, absorbed into the plant and carried throughout the tissues, being ingested by insects when they feed on the plant. Thus, it is effective against certain foliage-feeders that cannot be reached with spray applications. In general, chewing insects are affected more than sucking insects and insects that undergo complete metamorphosis are also generally affected more than those that do not undergo metamorphosis (Dubey, 2011).

2.1.2.2 Mode of action

The effects of azadirachtin on insects include feeding and oviposition deterrence, growth inhibition, and fecundity and fitness reductions (Schmutterer, 1990). Azadirachtin is a common example of a natural plant defense chemical affecting feeding, through chemoreception (primary antifeedancy), that consists in the blockage of the input from receptors that normally respond to phagostimulants, or from stimulation of specific deterrent cells or both (Dethier, 1982) and through a reduction in food intake due to toxic effects if consumed (secondary antifeedancy), where food intake is reduced after application of azadirachtin in ways which bypass the mouth part chemoreceptors (Mordue & Blackwell, 1993). The antifeedant effect is highly variable among pest species, and even those species initially deterred are often capable of rapid desensitization to azadirachtin (Bomford & Isman, 1996). Azadirachtin is a tetranortriterpenoid, structurally similar to insect hormones "ecdysones", its biological activity as ecdysone-blocker thus disturbing insect growth. This substance interferes with synthesis of the insect molting hormone, a-ecdysone, as well as other physiologically active neuropeptides in insects, producing a wide range of physiological and behavioral effects, such as anorexia. It also leads to sterility in female insects due to its adverse effects on ovarian development, fecundity, and fertility. For more information about the mode of action of neem, see Isman and Akhtar (2007) and Insect growth regulators below.

2.1.2.3 Safety

Azadirachtin is nontoxic to mammals. Different neem products were neither mutagenic nor carcinogenic, and they did not produce any skin irritations or organic alterations in mice and rats, even at high concentrations. The pure compound azadirachtin, the unprocessed materials, the aqueous extracts and the seed oil are the most safe to use as an insecticide to protect stored seeds for human consumption (Boeke et al., 2004). Ecologically, azadirachtin is non toxic to fish (Wan et al., 1996), natural enemies and pollinators (Naumann & Isman, 1996), birds, other wild life, and aquatic organisms as azadirachtin, breaks down in water within 50–100 h. It is harmless to non-target insects (bees, spiders, and butterflies). The effect of azadirachtin on natural enemies is highly variable (Hohmann et al., 2010, Kumar et al., 2010). Environmentally, azadirachtin induce no accumulations in the soil, no phytotoxicity and accumulation seen in plants, and no adverse effect on water or groundwater (Mehlhorn, 2011). Neem is sensitive to light and the half-life of azadirachtin is only one day (Kleeberg, 2006), leaving no residues on the crop and therefore are preferred over chemical pesticides. Azadirachtin is classified by the Environmental Protection Agency (EPA) in class IV.

2.1.2.4 Risk factors

The most critical adverse effects are reproduction disturbances, although these are often reversible. (Boeke et al., 2004). Neem pollen induces allergenic effect to some individuals (Karmakar & Chatterjee, 1994). Moreover, the oil can turn rancid (De Groot, 1991) and is easily contaminated with aflatoxins, so contaminated neem seeds with aflatoxin should not be picked from the ground but seeds that are greenish yellow in color should be picked from the trees or swept regularly under the tree (Gunasena & Marambe, 1998). Ecto-endo parasitoids vulnerable to neem but soil application could reduce negative side effects compared to plant spraying and hence improve selectivity (Kumar et al., 2010). Treating the host with neem before parasitism was less deleterious to wasp emergence, especially for *Trichogrammatoidea annulata* (Hohmann et al., 2010). For more details about

safety of neem, see Boeke et al. (2004), Mehlhorn (2011), Kumar et al. (2010), and Homanni et al. (2010).

2.1.2.5 Production

In order to produce and use efficacious neem pesticides, Saloko et al. (2008) reviewed some points that should be notes: neem leaf extracts are less effective than seed extracts due to lower azadirachtin content; neem preparations should be kept away from sunlight to avoid photodegradation of active ingredients by UV light, and formulations are better applied at dusk when sun is weak; sun screens such as Para Amino Benzoic Acid (PABA) could be added to reduce the photo-oxidation of azadirachtin by UV light.
Neem seeds contain 0.2% to 0.6% azadirachtin by weight, so solvent partitions or other chemical processes are required to concentrate to be 10% to 50% as in the technical grade material used for commercial production. World wide, there are over 100 commercial neem formulations such as Margosan-O, Bio-neem, Azatin, , Neemies, Safer's ENI, Wellgro, RD-Repelin, Neemguard, Neemark, and Neemazal. Formulations include emulsifiable concentrates (ECs), suspension concentrates (SCs), ultra low volume (ULV) formulations and granular formulations. The chemistry of azadirachtin was reached in 2007 and its synthesis was completed, see Morgan (2009). Azadirachtin and botanical preparations based on neem seed extracts are environmentally friendly pesticide and virtually non-toxic to mammals and wildlife, making them among the safest of all insecticides that used for integrated pest management and organic farming, For more details about neem, see Collins (2006), Isman & Akhtar (2007), Saloko et al. (2008), Gilbert & Gill (2010), Dubey (2011), and Regnault-Roger (2011).

2.1.3 Essential oils

Aromatic oils obtained through steam distillation of many plant families, ex. Myrtaceae, Lamiaceae, Asteraceae, Apiaceae, and Rutaceae are highly targeted for anti-insect activities against several insect orders. Approximately 3000 essential oils are known, and 10% of them have commercial importance in the cosmetic, food, and pharmaceutical industries. They are generally recognized as safe, GRAS, by the US Food and Drug Administration. Complete essential oils are more effective than individual constituents or even a combination of constituents.

2.1.3.1 Essential oil chemistry

The volatile components of essential oils can be classified into four main groups: terpenes, benzene derivatives, hydrocarbons, and other miscellaneous compounds. The major constituent of some oils are 8-cineole from rosemary (*Rosmarinus officinale*) and eucalyptus from (*Eucalyptus globus*); eugenol from clove oil (*Syzygium aromaticum*); thymol from garden thyme (*Thymus vulgaris*); and menthol from various species of mint (*Mentha* species). More information about essential oil chemistry is given by Isman (2006) and Tripathi et al. (2009).

2.1.3.2 Mode of action

Aromatic plants produce many compounds that act as ovicidal, larvicides, adulticides, insect arrestants and repellents or act to alter insect feeding behavior, growth and development, ecdysis (molting) and behavior during mating and oviposition.
Essential oils are lipophilic in nature and interfere with basic metabolic, biochemical, physiological, and behavioral functions of insects. Commonly, essential oils can be inhaled,

ingested or skin absorbed by insects. The rapid action against some pests is indicative of a neurotoxic mode of action, and there is evidence for interference with the neuromodulator octopamine (Enan, 2005) or GABA-gated chloride channels (Priestley et al., 2003).
Several essential oil compounds have been demonstrated to act on octopaminergic system of insects. Octopamine is a neurotransmitter, neurohormone, and circulating neurohormone – neuromodulator (Hollingworth, et al., 1984) and its disruption results in total break down of nervous system in insects. The lack of octopamine receptors in vertebrates likely accounts for the profound mammalian selectivity of essential oils as insecticides. Eugenol mimicked octopamine in increasing intracellular calcium levels in cloned cells from the brain of *Periplaneta americana* and *Drosophila melanogaster* (Enan, 2005). Consequently, octopaminergic system of insects represents a biorational target for insect control. Plant volatile oils have long been known to affect the behavioural responses of pests, with the monoterpenoid components appearing most useful as insecticides or antifeedants (Palevitch & Craker, 1994). LMW terpenoids may be too lipophilic to be soluble in the haemolymph after crossing the cuticle, and proposed a route of entry through the tracheae (Veal, 1996). Most insecticides bind to receptor proteins in the insect and, in doing so; they interrupt normal neurotransmission, which lead to paralysis and subsequently death. Recent evidence suggests that low-molecular-weight (LMW) terpenoids may also bind to target sites on receptors that modulate nervous activity. Ionotropic, γ-aminobutyric acid, GABA receptors, the targets of organochlorine insecticides lindane and dieldrin, are modulated by LMW terpenoids with vastly different structures (Priestley et al., 2006). Valuable appraisals about the mode of action are those of Price & Berry (2006), Isman (2006, 2010); Tripathi et al. (2009); and Dubey (2011). Some essential oils have larvicidal effect and the capacity to delayed development and suppress adults emergences and induce abnormalities during development of insects of medical and veterinary importance, Fig (1-5) (Khater, 2003; Shalaby & Khater, 2005; Khater & Shalaby, 2008; Khater & Khater (2009); Khater et al., 2009, 2011).

2.1.3.3 Repellent effect

Repellents are substances that provide a vapor barrier deterring the arthropod from coming into contact with the surface or flying to, landing on or biting human or animal skin. The use of insect repellent compounds dates back to ancient times as plant oils, smokes, tars, etc. were used to displace or kill insects. The use of repellents by travelers may reduce infection with local diseases in temperate areas. DEET (N,N-diethyl-m-toluamide) is a broad spectrum repellent and the most effective and persistent on skin. Unfortunately, it may cause environmental and human health risks (Pitasawat et al., 2003). Therefore, there has been an increase in search efforts for natural and eco-friendly repellents.

2.1.3.3.1 Plant- based repellents

Some plant-based repellents are comparable to, or even better than synthetics; however, essential oil repellents tend to being short-lived in their effectiveness due to their volatility. Nerio (2010) review some splendid ideas for improvement of repellency of essential oils. Repellency assays with essential oils were done for Diptera species, especially mosquitoes and to a lesser extent to coleopteran insects related to losses in stored food. Plants with strong smell, such as French marigold and coriander act as repellents and can protect the corps nearby. Several essential oil- producing plants have been widely studies, such as *Cymbopogon* spp., *Eucalyptus* spp., *Ocimum* spp., the osage

orange (hedgeapple) (*Maclura pomifera*), and catnip (*Nepeta cataria*). Several plant oils or their constituents have been commercialized as insect repellents in the past decade, such as soybean, lemon grass, cinnamon, and citronella. Neem oil, from *A. indica*, when formulated as 2% in coconut oil, provided complete protection (i.e. no confirmed bites) for 12 hours from *Anopheles* mosquitoes (Sharma et al., 1993). Essential oils have pronounced *In vitro* and *In vivo* pediculicidal activity as the number of lice infesting water buffaloes in Egypt was significantly reduced 3, 6, 4, and 6 days after treatment with the essential oils of camphor (*Cinnamomum camphora*), peppermint (*Mentha piperita),* chamomile (*Matricaria chamomilla*), and onion (*Allium cepa*), respectively. Surprisingly, the same oils repelled flies (*Musca domestica, Stomoxys calcitrans, Haematobia irritans,* and *Hippobosca equine*) infecting buffaloes for almost 6 days post-treatment. No adverse effects were noted on either animals or pour-on operators after exposure to the applied oils (Khater et al., 2009).

2.1.3.3.2 Metabolites reliable for repellent activity

Nerio et al. (2010) reviewed the repellent activity of essential oils which contributed to some metabolites, such as monoterpenes (α-pinene, cineole, eugenol, limonene, terpinolene, citronellol, citronellal, camphor, and thymol) against Mosquitoes (Yang et al., 2004) sesquiterpenes, β-caryophyllene, repellent against *A. aegypti*; phytol, a linear diterpene alcohol, against *Anopheles gambiae*; and phenylethyl alcohol, β-citronellol, cinnamyl alcohol, geraniol, and α-pinene, isolated from the essential oil of *Dianthus caryophyllum*, aginst ticks *(Ixodes ricinus).* In addition, cineole, geraniol and piperidine found in bay leaves (*Laurus nobilis*, Lauraceae) possess repellent properties towards cockroaches. Repellents may have an increasingly important role in eliminating insects from certain environments and essential oils could play a major role in new repellent technology. Valuable review on the repellent activity of essential oils are those of Tripathi et al. (2009), Isman (2010), Kumar et al. (2010), Nerio et al. (2010), Dubey (2011), and Maia & Moore (2011).

2.1.3.4 Fumigant

Today, The used fumigants, for instance, phosphine, methyl bromide, and DDVP (2,2-dichlorovinyl dimethyl phosphate) do have adverse effets. Phosphine is the major cause of suicidal deaths in India. Methyl bromide has ozone-depleting potential (UNEP, 2000) and DDVP has a possible human carcinogen potential (Lu, 1995). Thus, there is an urgent need for development of safe alternative that have the potential to replace the toxic fumigants against pests attacking grains, dry stored food, and other agricultural products. The active principles are monoterpenes, sesquiterpenes and their biogenically related phenols. In addition to direct toxicity to insects, many of these substances are eterrents or repellents. Essential oils of *Artemisia species, Anethum sowa, Curcuma long,* and *Lippia alba.* Clove, rosemary, thyme, eucalyptus and various mint species have demonstrated contact and fumigant toxicity to a wide spectrum of insects, including human head lice (Toloza et al., 2008). Isolates like d-limonene, carvones and 1,8-cineole have been well documented as fumigants. The exact mode of action of these oils as fumigant is unknown, but the oils mainly act in the vapour phase via respiratory system. Physical properties of essential oils such as high boiling point, high molecular weight and low vapor pressure are barriers for application in large scale fumigation. For more details about fumigants, see Tripathi et al., (2009), Isman (2010), and Dubey (2011).

2.1.3.5 Commercialization

Although essential oils are effective when freshly applied, their protective effects usually dissipate relatively quickly. In their review, Nerio et al. (2010) discussed methods to access repellency effects, the synergistic phenomena of such oils and some novel ideas to increase the repellent efficiency. Some fixative materials such as liquid paraffin, vanillin, salicyluric acid, mustard, and coconut oils have been used. Formulations based on creams, polymer mixtures, or microcapsules for controlled release, resulted in an increase of repellency duration. Still, essential oils can be incorporated with polymers into sheets and attractant adhesive films with essential oils were prepared to control insects in agriculture and horticulture. Novel ideas are needed to be explored for better commercialization of essential oil- based pesticides. Several essential oil constituents are already in use as an alternative to conventional insecticides, such as Green Ban® (containing oils of citronella, cajuput, lavender, safrole free sassafrass, peppermint, and bergaptene free bergamot oil); Buzz Away® (containing oils of citronella, cedarwood, eucalyptus, and lemongrass); ValeroTM, a miticide/fungicide for use in grapes, berry crops, citrus, and nuts; and CinnamiteTM, an aphidicide /miticide/fungicide for glasshouse and horticultural crops. The last two products are based on cinnamon oil, with cinnamaldehyde (30% in EC formulations) as the active ingredient. In addition, d-limonene is an active ingredient of commercially available flea shampoos, plus pulegone and citronellal are used as mosquito repellents.

2.1.3.6 Safety of essential oils

Currently, the US Environmental Protection Agency (US EPA) has registered citronella, lemon, and eucalyptus oils as insect repellent ingredients for application on the skin. Using essential oils or some of their products could cause dermatitis, they should be rubbed on a small portion of skin to determine if there will be an allergic reaction before treating your whole body. The most attractive aspect of using essential oils and/or their constituents for pest control is their favorable mammalian toxicity because many essential oils and their constituents are commonly used as culinary herbs and spices. Many of the commercial products including essential oils are included on the GRAS list fully approved by FDA and EPA in USA for food and brevarage consumption (EPA, 1993). Some of the purified terpenoid constituents of essential oils are moderately toxic to mammals, but, with few exceptions, the oils themselves or products based on oils are mostly nontoxic to mammals, birds, and fish. Although natural enemies are susceptible via direct contact, predators and parasitoids reinvading a treated crop one or more days after treatment are unlikely to be poisoned by residue contact as often occurs with conventional insecticides. Owing to their volatility, the oils and their constituents are environmentally nonpersistent, with outdoor half lives of\24 h on surfaces, in soil and in water (Isman el al., 2011). There is no harvest restrictions or worker re-entry restrictions for treated crops; they are compatibile with biological control agents and indigenous natural enemies of pests, and they bring about reduce risks to honeybees and other foraging pollinators. For additional information about safety of essential oils, see Isman (2006, 2010), Tripathi et al. (2009), Nerio et al. (2010), and Regnault-Roger (2011). Because many conventional pesticide products fall into disfavour with the public, botanical-based pesticides should become an increasingly popular choice for pest control.

2.2 Insect growth regulators

Insect growth regulators (IGRs) are chemical compounds that alter growth and development in insects. They don't directly kill insects, but interfere with the normal

mechanisms of development, resulting in insects dying before they reach adulthood. IGRs are classified into two general categories based on mode of action: chitin synthesis inhibitors and substances interfering with the action of insect hormones.

2.2.1 Chitin synthesis inhibitors

Chitin synthesis inhibitors (CSIs) affect the ability of insects to produce new exoskeletons when molting. They act on the larval stages by inhibiting or blocking the synthesis of chitin which represent 30-60% of the insect exoskeleton structure. They also increase egg mortality. CSIs include conventional benzoylureas, triazine/pyrimidine derivatives, and buprofezin.

2.2.1.1 Benzoylphenylurea

Typical effects benzoylureas or benzoylphenylurea (BPUs) on developing larvae are the rupture of malformed cuticle or death by starvation. BPUs act as ovicides, reducing the egg laying rate or hindering the hatching process by inhibiting embryonic development or failure of hatchability. Commertial porducts of BPUs include diflubenzuron (Dimilin®, Adept®, Micromite®); triflumuron (Alsystin®); teflubenzuron (Nomolt®, Dart®), hexaflumuron (Trueno®, Consult®); chlorfluazuron (Atabron®); flufenoxuron (Cascade®); and flucycloxuron (Andalin®). Among the newer benzoylureas only hexaflumuron (1993) and novaluron (2001) have been registered by EPA. Studies with diflubenzuron, the most investigated BPU, revealed that it alters cuticle composition, especially inhibition of chitin, resulting in abnormal endocuticluar deposition that affects cuticular elasticity and firmness, and cause abortive molting. Diflubenzuron (Dimilin® El –Delta Company, Egypt) is highly effective in controlling mosquitoes, *Culex pipiens* than house flies, *Musca domestica*. LC 50 values were 1.26 and 1000 ppm, respectively. All treated late 3rd and early 4th larvae of *C. pipiens* (concetnraions: 0.04 - 40 ppm) were eventually died as Dimilin® prolonged the larval durations (11.9 days vs. 4 days in the control group) and increased larval abnormalities (46.7%). Such abnormalities were larvae with transparent cuticle, splitting of cuticle, and pharate pupae (Fig. 4). It induces pupal abnormalities as well (Fig. 5). Treatment of of *M. domestica* with the same product (at 1ppm) induced larval and pupal malformations reached 23.3 and 56.5%, respectively, and reduce adult emergence (66.7%). Abnormalities of *M. domestica* include small, shrunken, macerated larvae and larvae with week cuticle as well as distorted puparia and failure of adult eclosion (Khater, 2003) (Fig. 1-3).

2.2.1.2 Triazine/pyrimidine derivatives

2.2.1.2.1 Cyromazine

Cyromazine (Larvadex ®, Trigard®), a triazine, is a potent CSI and it is selective toward dipterous species and fed to poultry or sprayed to control flies on animals, in manure of broiler and egg producing operations. It controls blowfly infesting sheep and persist for up to 13 weeks (O'Brien & Fahey, 1991) after a single pour-on application, or longer if applied by dip or shower. Moreover, it is used as a leafminers spray in vegetable crops and ornamentals. Cyromazine may inhibit growth or expansion of the body wall (or both) sufficiently to prevent normal internal growth, producing the observed symptoms and leading to abnormal development. The presence of three resistant house fly populations to cyromazine in Brazilian poultry farms strongly suggests that the operational aspects of larvicide use are important for the development of resistance. Cyromazine is applied as a feed-through, both in Brazil and in the USA, where resistance has already been documented. However, in Denmark, where it was approved only as a topical manure spray, no case of resistance has yet been detected (Pinto & do Prado, 2001).

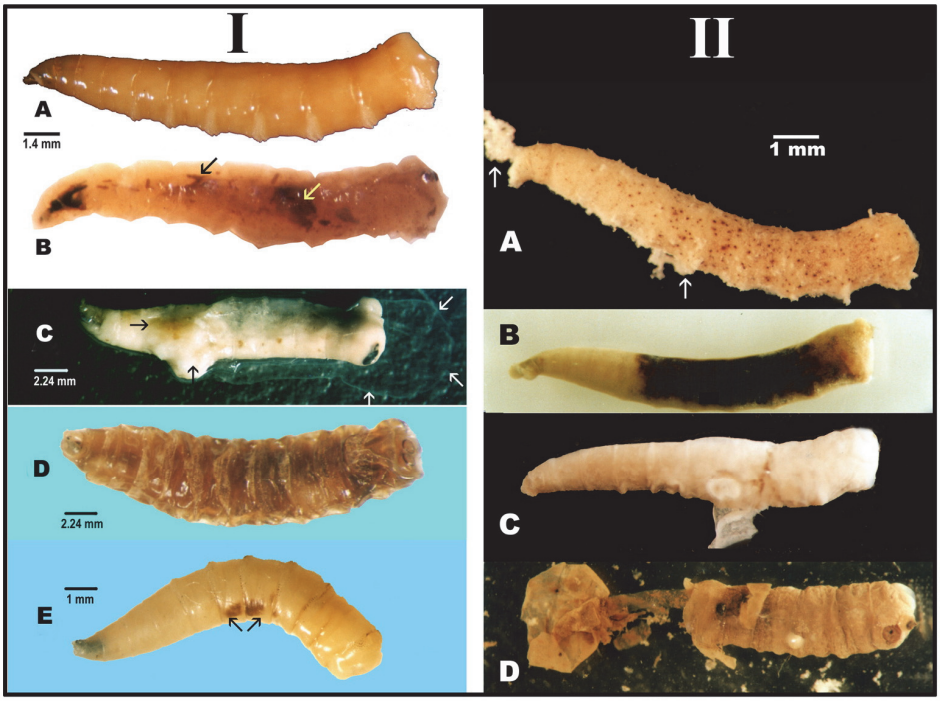


Fig. 1. Morphological malformations of larvae of house flies. I. A. Normal larva. B-E. Malformed larvae, treated with essential oils and insect growth regulators showing signs of pigmentation. C. Macerated larva with week transparent cuticle. II. Larvae infected with fungi. A. Red Pin -point pigments all over the larval body with apparent fungal growth (arrow heads). B. Larva with diffuse blackish pigmentation. C. Larva with an ulcer in the middle. D. Ulcerated and macerated larva with white nodules and fungal growth.

2.2.1.2.2 Dicyclanil

Dicyclanil (ZR ®, ComWin ®), a pyrimidine derivative, is highly active against dipteran larvae and available as a pour-on formulation for blowfly control in sheep in Australia and New Zealand providing up to 20 weeks' protection (Bowen et al., 1999). On the whole,

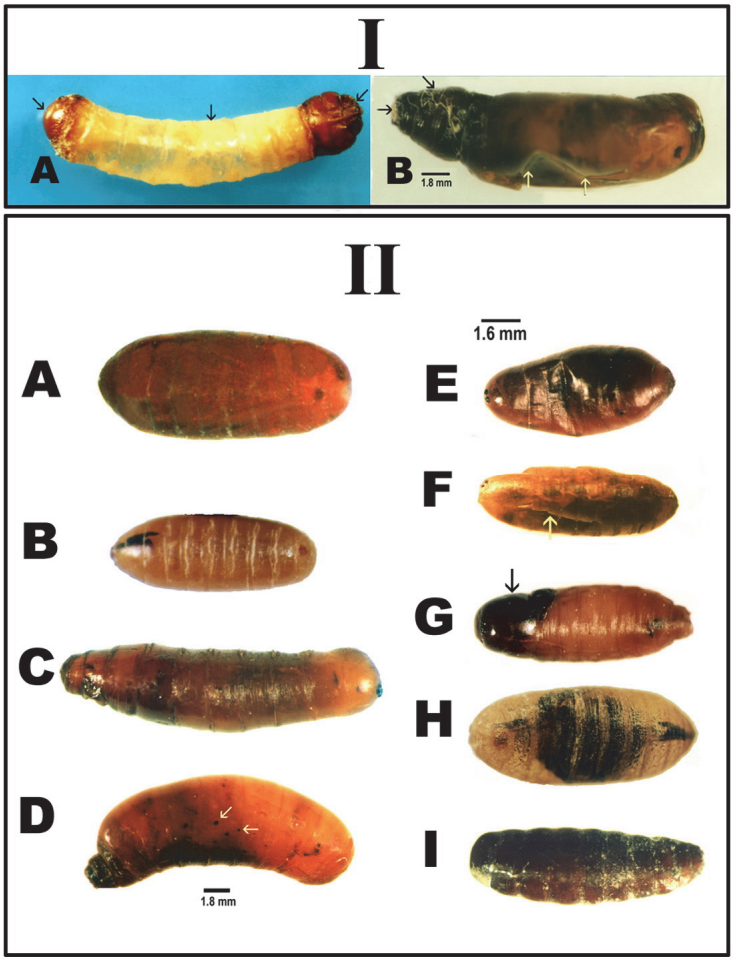


Fig. 2. Pupal abnormalities of house flies: I. Larval pupal- intermediates: A. The anterior and posterior ends as pupae (left and right arrows), while the rest of the body as larva (middle arrow). B. Fungal growth anteriorly (left arrow) and cracked pupal case at the middle (lower arrows). II. Pupae: A. Normal pupa. B. Small pupa with visible cephalopharyngeal skeleton. C. Larviform pupa, pigmented with small dark spots at the intersegmental regions. D. C-shaped pupa with anterior constriction and small patches of black pigments (arrow heads). E. Distorted puparia. F. Pupa with a groove (arrow head). G. Blackish posterior end (arrow head). H. Transparent puparia. I. Hyphal growth appears on a small puparia. Adapted form Khater (2003).

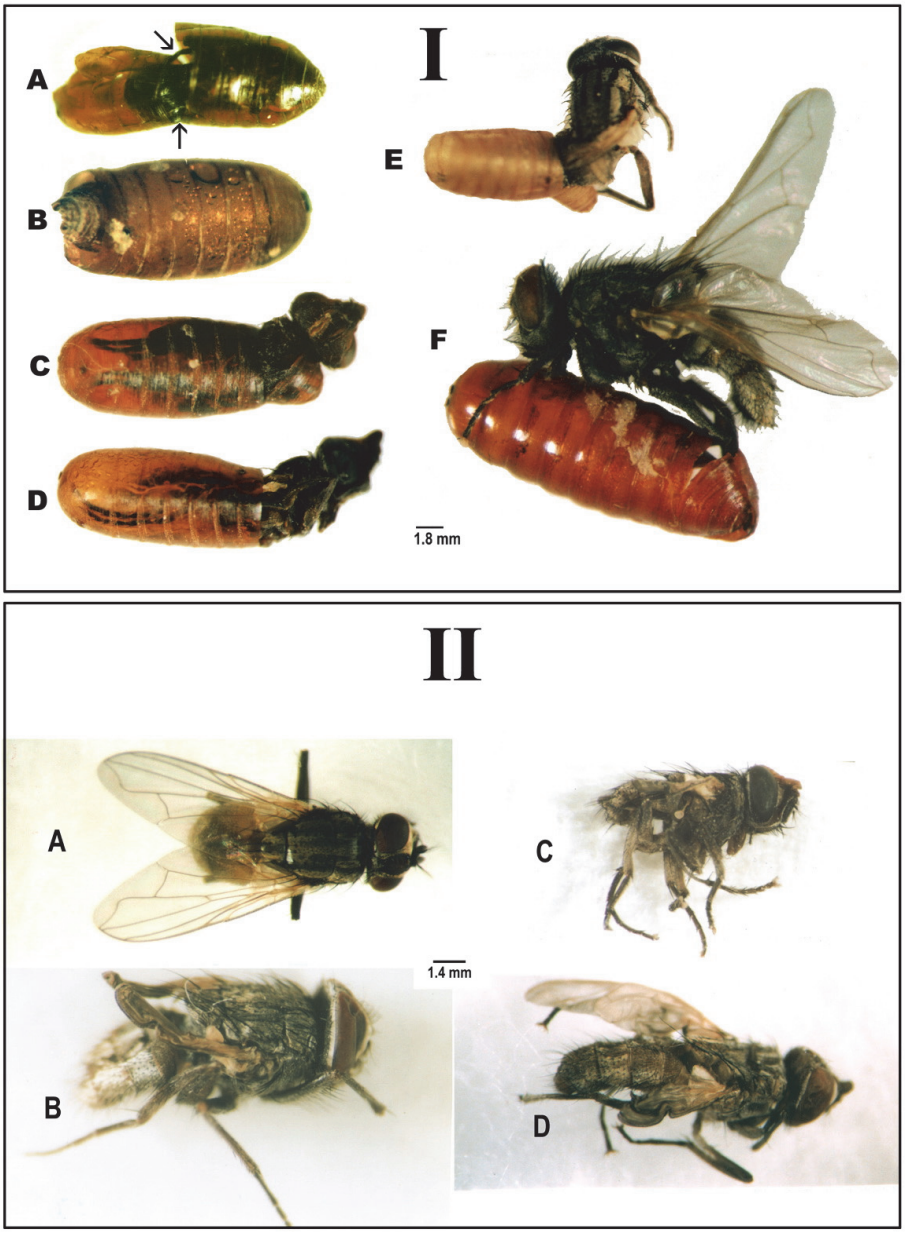


Fig. 3. Morphological abnormalities of house flies. I. (A-F) Failure of adult eclosion. II. Adults (A. Normal adult, B. Crumpled adult with poorly developed wing and legs, C. Small adult, D. Elongated adult with deformed wing, abdomen, and legs. Adapted from Khater (2003).

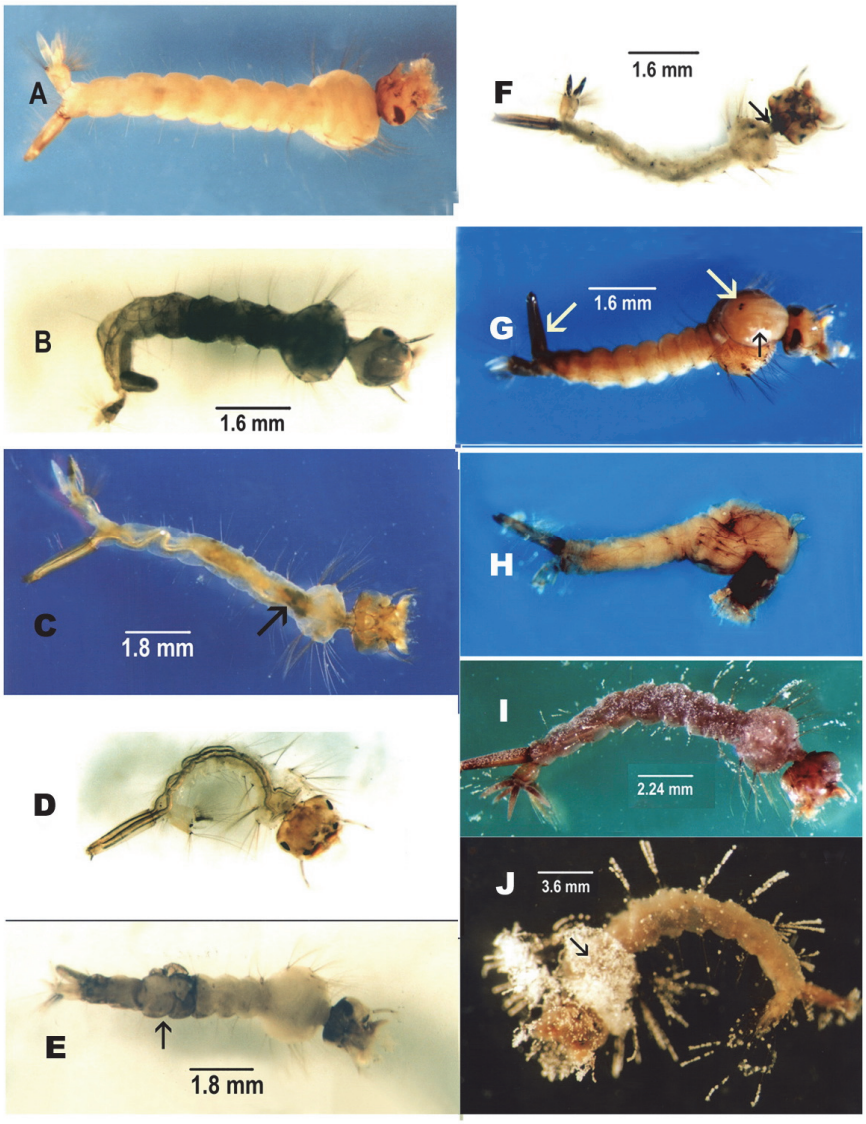


Fig. 4. Morphological abnormalities of mosquito larvae: A. Normal larva, B. Pigmented larva, C-F. Larvae with deformed cuticles, G. Larva with an opaque swelling on the thorax and black coloration at the posterior end, H. Pharate pupa (prepupa), I, J. larvae show symptoms of mycosis. Adapted from Khater (2003).

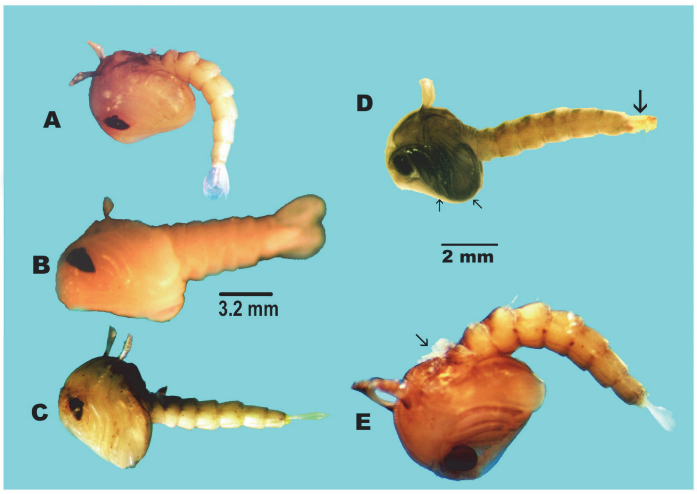


Fig. 5. Pupal abnormalities of mosquitoes: A. Normal pupa. B. Albino pupa. C. Elephantoid pupa, with enlarged cephalic region and extended abdomen. D. Black cephalothorax (lower arrows) and extended abdomen with transparent posterior end (upper arrow) and no anal gills. E. Pupa with apparent fungal growth (arrow head). Adapted form Khater (2003).

most CSI compouns are very potent against a variety of different pests, especially lepidopterous insects and whiteflies. They are harmless or exert little adverse effect on bees, predators, or parasitoids (Tomlin, 2000) which renders them acceptable for inclusion in IPM programs.

2.2.2 Substances interfering with the action of insect hormones

Growth and development of insects are regulated by hormones: prothoracicotrophic hormones (PTTH) (brain hormone), ecdysteroids, and juvenile hormones (JH). PTTH controls the secretion of the molting hormone (ecdysone) from the prothoracic gland. Ecdysone is responsible for cellular programming and, together with JH, initiating for the molting process. High secrection of JH from the corpora allata allows the epidermis to be programmed for a larval molt, or else it is programmed for metamorphosis. JH is virtually absent in the pupae, but is present in adults to serve some functions in reproduction. JH suppresses pupation and induces vitellogenesis during the reproductive stage of the insect (Eto, 1990).

2.2.2.1 Ecdysteroid agonist

Bisacylhydrazine (BSH), a newer class of IGRs, is ecdysone agonists or disruptors with molting hormone activity. BSHs include chromafenozide (Virtu ®), Tebufenozide (Mimic ®, Confirm ®), Halofenozide (Mach-2 ®), and Methoxyfenozide (Intrepid ®). They have a greater metabolic stability than the insect steroid molting hormone 20-hydroxyecdysone (20E) *In vivo* (Retnakaran et al., 1995). They are toxic after ingestion or exposure to higher doses of topical application. Ingestion of BSH creates hyperecdysonism in susceptible insect, including molting events. The effect starts with feeding inhibition within 3-14 h (Retnakaran et al., 1997), which is very important for preventing further crop damage. In the mean while,

larvae become moribund, slip their head capsule, and the hind gut may be extruded in extreme cases. Synthesis of new cuticle begins and apolysis of the new cuticle form the old one takes place. The new cuticle is not sclerotized or tanned. Consequently, the food intake by larva is prevented as the mouthparts become soft and mushy. Larval death is due to incomplete molting, starvation, and desiccation due to hemorrhage. Both Tebufenozide and methoxyfenozide, selectively toxic to Lepidopteran pests, have been classified by US EPA as reduced risk pesticides. It worth to mention that azadirachtin in neem tree acts as ecdysone blocker and disrupt insect growth, see botanical insecticides above. In general, BSHs have insect selectivity, reduced risk ecotoxicology, and mammalian profile as they have new mode of action, therefore, they are ideally integrated in IPM programs.

2.2.2.2 Juvenile hormone analogues

The juvenile hormone analogues (JHA) mimic the activity of naturally occurring JHs and prevent metamorphosis to the adult stage. JH is one of the most pleiotropic hormones known and functions in various aspects such as embryogenesis, molting and metamorphosis, reproduction, diapause, communication, migration/ dispersal, caste differentiation, pigmentation, silk production, and phase transformation. The major function of JH is the maintenance of larval status or the so- called juvenilizing effect, See Tunaz (2004). JHAs are highly effective at the beginning stage of metamorphosis and embryogenesis, for instance, freshly ecdysed last larval instars, freshly ecdysed pupal instars, and deposited eggs. Thus embryogenesis is disrupted when young eggs are treated with JHAs. Application to early last instar larvae would result in the development of supernumerary instars, whereas treatment at the later stage would result in abnormal pupation and development of larval-pupal mosaics or intermediates (Khater, 2003). Treatment of of *M. domestica* and *C. pipiens* with pyriproxyfen (Sumilarv ® Somitomo Co.) interferes with normal metamorphosis and results in various developmental abnormalities including larval pupal intermediates that do not survive (Fig. 1-5); Lower concentrations significantly prolonged larval and papal durations, 10.5 and 11 days (at conc. 0.1 ppm) and 9.9 and 15.1 days (at conc. 4 ppm), respectively, than those of the control (3, 5.7 days and 3.9, 1.1 days, respectively) (Khater, 2003). Methoprene (Altosid ®, Apex ®, Pharorid ®, Precor ®), the first compound introduced into the market, is a terpenoid compound with very low mammalian toxicity and is regularly used for flea control. It is sensitive to light. Thus, It has been used extensively and successfully in indoor environments and on pets in the form of collars, shampoos, sprays, and dips and also as a feed through larvicide for hornfly, *Haematobia,* control on cattle (Graf, 1993). Other IGRs available for use against household and agricultural pests are pyriproxyfen and fenoxycarb. Fenoxycarb (Logic®, Award®, Comply®, Torus®) is a carbamate stomach insecticide that has also JH-type effects when contacted or ingested by various arthropod pests, e.g., ants, roaches, ticks, chiggers and many others. Some JHAs are of plant origin, such as Juvenoids, isolated form plants, the "paper factor" form the balsam fir (*Abies balsamea*) and Juvocimenes from the sweet basil plant (*Ocimum basilicum*) (Bowers & Nishida, 1980). Morphogenetic control is effective for controlling insects that are pests as adults, like most insects of medical and veterinary importance, ex. mosquitoes, nuisance flies, and fire ants. They are not effective against lepidopteran agricultural pests because the larval stage is responsible for plant destruction. Many JH analogs (or mimics) (JHAs) are attractive candidates for pest control because of the ease of their synthesization and their pest selectivity than those of other peptide and steroid hormones (Eto, 1990).

2.2.2.3 Antijuvenile hormones

Antijuvenile hormones, anti- JHs, are very effective pest control agents as they prevent JH production, facilitate JH degradation, or destroy corpus allatum. Intoxicated newly emerged larvae with anti- JH would create miniature pupae, thus abbreviating the distructive part of insect life cylce. Some of these plant-derived substances actually serve to inhibit the development of insects feeding and protecting the host plant. These are referred to broadly as antijuvenile hormones, more accurately, antiallatotropins, or precocenes. Although the mode of action of the precocenes is still unclear, it is known that they depress the level of juvenile hormone below that normally found in immature insects. As anti-JHs is competing with JH in binding to the JH receptors or to the JH carrier proteins, injuring the corpora allata cells, or interfering with JH biosynthesis (Leighton et al., 1981), other JHAs may also function as anti-JHs, such as ETB [ethyl 4-(2- pivaloyloxybutyloxy)-benzoate], which showed JH agonist and antagonist activities in *Manduca sexta* larvae as it compete with JH at the receptor site and become feedback inhibitors of JH biosynthesis (Staal, 1986).

2.2.3 Safety

Several IGRs are registered by the EPA, such as methoprene (Altosid ®) which is used as a grain protectant, as mosquito growth regulator; Precor ® for indoor control of dog and cat fleas; Hydroprene (Gentrol ® , Mator ®) for use against cockroaches, and stored grain pests; and kinoprene (Enstar II ®), which is effective against aphids, whiteflies, mealybugs, and scales (both soft and armored) on ornamental plants and vegetable seed crops grown in greenhouses and shadehouses. Although some insects acquired resistance against some IGR- based products, IGRs are typically "safer" to use around humans, pets, and natural enemies than conventional insecticides and acaricides. IGRs are effective when applied in very minute quantities and generally have few or no effects on humans and wildlife. They are, however, nonspecific, since they affect not only the target species, but other arthropods as well. For more information, see Taylor (2001), Tunaz (2004), Ware & Whitacare (2004), Collins (2006), and Gilbert & Gill (2010).

2.3 Pheromones

Pheromones are a class of semiochemicals that insects and other animals release to communicate with other individuals of the same species. Such behavioral chemicals, range from small hydrophobic molecules to water-soluble peptides, leave the body of the first organism, pass through the air (or water) and reach the second organism, where they are detected by the receiver. Signalling chemicals play an essential role in arthropod life cycles. They provide the means whereby mates, host and oviposition sites are located and recognized (Mordue Luntz, 2003). Pheromones may signal various information. Long-lasting pheromones allow marking of food sources or territorial boundaries. Other signals are very short-lived and provide an immediate message, such as a brief period of reproductive readiness or short-term warning of danger.

2.3.1 Uses of pheromones

There are five principal uses for sex pheromones: population monitoring, mass trapping of insects, movement studies, detection of exotic pests and, mating disruption. Such disturbance is very important in reducing the population density of pests as synthetic

pheromone is dispersed into the field and the false odor plumes attract males away from females that are waiting to mate, thus reduce the population density of the pests. In contrast to the previous benefits, the high degree of selectivity may be a barrier to large-scale implementation where secondary pests become a problem as insecticides are eliminated (Walker & Welter, 2001). Other obstacles include the following: the lack of an identified pheromone for some pest species, high development and production costs, requirements for specialized application techniques or equipment, and the need to supplement the pheromone program in high pest-pressure situations (Welter et al., 2005). Large-scale implementation projects have yielded significant reductions in pesticide use while maintaining acceptably low crop-damage levels. Pheromones manipulate the behavior of insect pests. With these non-toxic and biodegradable chemicals, insects can be lured into traps or foiled into wasting energy that they normally need for locating food and mates. Pheromones are species-specific chemicals that affect insect behavior and they are not toxic to insects or other non- target organisms, creating opportunities for the biological control of other pest species. They are attractive in extremely low doses and used to bait traps or confuse a mating population of insects. Other advantages include negligible health risks, no accumulation in wildlife or groundwater, limited impacts on other management practices; manage insecticide resistance, and a more rapid registration process. Pheromones can play an important role in integrated pest management for medical or veterinary, structural, agricultural, landscape, and forest pest problems. Pheromone programs are most effective with low to moderate population densities. Because of some difficulties with high populations of pests, these programs should not be viewed as stand-alone strategies but rather as one tactic within a suite of integrated pest management options (Welter et al., 2005). For more information about pheromones, see; Cooping & Menn (2000), Witzgall (2001), Leal et al. (2003), Bray et al. (2009), and van Emden & Service (2011).

2.4 Photosenitizers

Development of new, ecologically safe technologies to control insect pest populations is of great importance. Photosensitizers are activated by illumination with sunlight or artificial light sources, have been shown to be accumulated in significant amounts by a variety of insects when they are administered in association with suitable baits. The subsequent exposure of such insects to UV/visible light leads induces lethal photochemical reactions and death. The most famous photosensitizers are xanthenes (e.g. phloxin B) and porphyrins (e.g. haematoporphyrin) which appear to be endowed with the highest photoinsecticidal activity. In particular, porphyrins absorb essentially all the UV/visible light wavelengths in the emission spectrum of the sun; hence they are active at very low doses. Photoactive compounds usually used for photosensitization might be effective as pesticide agents, with low impact on the environment, being non-toxic and not mutagenic, see Ben Amor & Jori (2000), Mangan & Moreno (2001), Ragaei & Khater (2004), Lukšienè etal.(2007), and Awad et al. (2008) for more fine points.

2.5 Inorganics and organic acid

Several inorganic substances are well know with their insecticidal effect, such as potassium silicate, diatomaceous earth (DE, diatomite or kieselgur), mineral oils, sulfur, boric acid, sodium borate, silica gels, kaolin clay, and soap spray. For more details about inorganic and their uses, see Ware & Whitacare (2004), and Collins (2006). It worth to mention that peracetic

acid, an organic acid, $C_2H_4O_3$, has strong acaricidal effect which was discovered for the first time (Khater & Ramadan, 2007). PPA had a great potential as acaricide against the cattle tick, *Boophilus annulatus*, and the fowl tick, *Argas persicus, In vitro.* Two minutes after treatment with 0.5%, PAA induced 100% mortality of both tick species and LC50 values for cattle and fowl ticks, after treatment for 30 min, were 0.06 and 0.05%, respectively. Following treatment with 0.25%, the LT50 values were 0.02 and 3.12 min, respectively. Furthermore, the detrimental effect of PAA against cattle tick extended beyond the adult stage, it significantly prolonged the preoviposition period, shortened the oviposition period, and decreased the mean number of the layed eggs, and such parameters were 14.75 and 6.57 days, and 457.50, respectively, after treatment with 0.25% of PAA. Therefore, PAA is highly effective when used at lower doses and short exposure time. The high speed of killing ticks is very important for avoidance of the hazard ensued by pathogen transmission in the course of delayed mortality caused by the currently used acaricides (Khater & Ramadan, 2007). PAA is highly effective against lice, *In vitro* and soft tick (*In vivo*), (Khater, H.F., Unpublished data).

3. Biological control

Biological control is the reduction or protection of pest populations by natural enemies which include four categories: microbes or pathogens (such as viruses, bacteria, protozoa, and fungi); entomopathogenic nematodes; predators (such as lady beetles and lacewings); and parasitoids (wasps and some flies). Protozoa, predators, and parasitoids are out of the scope of this chapter. Natural enemies are responsible for natural suppression of pest population. The first successful large scale microbial control application using coidiospores of the fungus *Metarhizium anisopliae* was carried out in the Russian Ukraine against the beet weevil, *Bothynoderes punctiventris* (Metchnikoff, 1879).

3.1 Virus

Entomopathogenic viruses are obligate disease-causing organisms that can only reproduce within a host insect. Among the fifteen or more families of viruses of invertebrates, it is mainly those having virus particles (virions) occluded within a proteinaceous matrix, an occlusion body (OB), have been used successfully in controlling pest populations. Such families are Entomopoxviridae (Entomopoxviruses, EPVs), Reoviridae (cypoviruses, CPVs), and Baculoviridae (Baculoviruses, BVs) (Lacy & Kaya, 2007). Only BVs have been used as pesticides (Szewczyk et al., 2009, 2011). In the past, the classification of the family Baculoviridae was based on virus morphology. It was divided into two genera: the Nucleopolyhedrovirus (NPVs) and the Granulovirus (GVs). A new division on the basis of comparison of genomic sequences indicate that virus phylogeny followed more closely the classification of the hosts than the virion morphological traits. Accordingly, family Baculoviridae contains four genera: Alphabaculovirus (lepidopteranspecific NPVs), Betabaculovirus (lepidopteran-specific GVs), Gammabaculovirus (hymenopteran-specific NPVs), and Deltabaculovirus (dipteran-specific NPVs) (Jehle et al., 2006). Although wider use of BVs as commercial insecticides was restricted because of their slow killing action and difficulties in large scale production, a very successful project was carried out in Brazil (Moscardi, 1999); over 2.0 million hectare of soybean had been already controlled annually by velvetbean caterpillar BV. Consequently, many countries have increased the area of fields and forests protected by BV pesticides.

3.1.1 Life cycle and viral stability

Virual infection begins in the insect's digestive system after consumption of plant material with viral particles. The virus spread though out the body but the digestive system is among the last part to be destroyed, so the insects usually continue to feed until they die leading to economic loss. See Lacey & Kaya (2007) and McNiel (2010) for more information about biology and ecology of viruses. The virus manipulates the behavior of infected larvae for its own dissemination. For example, larvae of the cabbage moth, *Mamestra brassicae* infected with NPV moved up to five times more than their healthy counterparts during the middle stages of infection (Vasconcelos et al., 1996b). Adult insects can also disperse viruses via vertical and horizontal transmission. Understanding the biology and ecology of viruses is crucial for optimizing pest control strategies of exotic and genetically modified organisms, see Lacy & Kaya (2007) and Szewczyk et al. (2009, 2011) for more details. UV protectants are very important for stability of the viral product, stilbene fluorescent brighteners (e.g. Phorwite AR ®, Blankophor ®, and others) induce the best results. Plant metabolites as peroxidases generate free radicals (Hoover et al., 1998) inactivate BVs. As a result, addition of free radical scavengers such as mannitol or enzyme superoxide dismutase to BV preparations can reduce such inactivation (Zhou et al., 2004). Tillage buries virus particles in the soil, thus good agricultural practices can reduce viral persistence between seasons.

3.1.4 Products

Some BVs are produced as commercial products, mainly for caterpillars, such as Gemstar LC (NPV of *Heliothis/Helicoverpa* spp. e.g., corn earworm, tobacco budworm, cotton bollworm); Spod-X LC (NPV of *Spodoptera* spp. e.g., beet armyworm); CYD-X and Virosoft CP4 (GV of *Cydia pomonella*, the codling moth); and CLV LC (NPV of *Anagrapha falcipera*, the celery looper). Successful infections can perpetuate the disease outbreak making repeat applications within a season unnecessary.

3.1.5 Safety

Members of BVs are regarded as safe to vertebrates. Their specificity is usually very narrow, often limited to single insect species. Regarding beneficial arthropods, immature larvae of parasitoids in infected hosts may die not due to virus infection, but relatively to premature loss of the host or to variation in quality of the host. Virus can provide safe, effective and sustainable control of a variety of insect pests as a part of a varied IPM programs, see El-Husseini (2006) and Szewczyk et al. (2009). Entomopathogenic viral agents are ideal for pest management purposes due to their specificity, dispersal capacity, and self propagation. Viruses are the most environmentally acceptable components of direct and integrated management regimes. Accordingly, there is global interest in use of viral agents. Valuable reviews on entomopathogenic viruses are those of Szewczyk et al. (2009, 2011), Lacey & Kaya (2007), McNiel (2010), Ahemed et al. (2011), and Singh et al. (2011).

3.2 Bacteria

The insecticidal bacterium, *Bacillus thuringiensis* (Bt) is a widely occurring gram-positive, spore-forming soil bacterium that produces parasporal, proteinaceous, crystal inclusion-bodies during sporulation. Bt has been the most successful commercial microbial insecticide, and also has been the subject of the overwhelming majority of genetic engineering studies to improve efficacy (Federici, 2010; Lacey & Kaya, 2010; Mehlhorn, 2011). Bt is actually a

complex of bacterial subspecies that occur in soil, leaf litter, leaf surfaces, insect feces, and as a part of the flora in the midguts of many insect species. There are several insecticides based on various sub-species of *Bacillus thuringiensis* Berliner (*Bt*), such as *B thuringiensis israelensis* (Bti), with activity against mosquito larvae, black fly (simuliid), fungus gnats, and related dipterans species; *B thuringiensis kurstaki* (Btk) and *B thuringiensis aizawai* (Bta)with activity against lepidopteran larval species; *B thuringiensis tenebrionis* (Btt), with activity against coleopteran adults and larvae; and *B thuringiensis japonensis* (Btj) strain *buibui*, with activity against soil-inhabiting beetles.

3.2.1 Mode of action

The insecticidal properties of Bt are largely a function of the presence of extra-chromosomal plasmids in the cell. These carry genes such as *cry* genes that encode a diverse array of these protein crystalline inclusion bodies which are toxic to insects. Upon ingestion by an insect, the crystal proteins (Cry) are solubilised and the insect gut proteases convert the original protoxin into smaller toxins. These hydrolysed toxins bind to the insect's midgut cells at high-affinity and specific receptor binding sites where they interfere with the potassium ion dependent, active amino acid symport mechanism decreasing absorption of minerals and nutrition from midgut and finally death of the columnar cells. This disruption causes the formation of large cation-selective pores that increase the water permeability of the cell membrane. A large uptake of water causes cell swelling and eventual rupture, disintegrating the midgut lining. Different toxins bind to different receptors with different intensities and this explains the selectivity of different Bt strains in different insect species (Baum et al., 1999; Cooping & Menn, 2000; Lacey & Kaya, 2007; Federici, 2010). Delayed larval mortalities (2–48 h) is caused by the crystal inclusions which have to be ingested and then processed within the insect's gut. Biological control products may contain the endotoxins plus live bacterial cells. The toxin stops feeding, this action hinder further damage caused by the feeding larva, and do not directly kill insects, but young larvae may starve to death and may die from bacterial infection over a longer period. Some commercial products contain Bti crystal proteins and spores, such as Bactimos and VectoBac (Valent BioSciences). VectoBac, Bti, (12 AS, Wady El- Niel for agricultural development Co. Egypt) is highly effective against *C. pipence* than *M. domestica*, LC50 values were $1x10^{-5}$ and $3.86 x10^{3}$ spores/ml, respectively, and LC 90 values were 0.04 and 37.28×10^{3} spores/ ml, respectively. Survived mosquito larvae died as pupae (Khater, 2003). Bti is a practical substitute to organophosphate insecticides, but it is unsuitable for application to environmentally sensitive water bodies. *Bacillus sphaericus* control mosquito larvae, particularly *Culex* and anopheline spp., especially those breeding in polluted water. It controls also black fly, *Simulium* sp., the vector of river blindness disease. Bs is effectively controlled *C. pipiens* in Egypt (Ragaei et al., 2004). It is widely used in Europe and Africa. Registered *B. sphaericus* product is Vectolex CG (Valent BioSciences). Recent studies in Kenya have shown that at least in some areas, biting rates by *Anopheles gambiae* can be reduced by more than 90% by using a combination of existing commercial formulations of Bti and Bs (Filinger & Lindsay, 2000).

3.2.2 Bacterial toxins

The use of insect-specific toxins from Bt and Bs is forming an increasingly component of biological control strategies. The protein crystals (protoxins) contain several toxins which

are classified according to their insecticidal activity and molecular relationship into four major gorups (Höfte and Whiteley, 1989): Cry I toxins, active against larvae of lepidopterans; Cry II toxins, active against larvae of lepidopterans and dipterans; Cry III toxins, active against larvae of coleopterans (Chrysomelidae); and Cry IV toxins, active against larvae of nematoceran flies. Cry toxins bind to glycoprotein or glycolipid receptors. The toxins can also be further categorized into subclasses, ex. CryIA etc. The genes of the toxins are named as the toxins but without capital letters and in italic (e.g., *cry*IV). Cytotoxins (Cyt) found in strains toxic to larvae of nematoceran flies and coleopterans. The CytA protein binds to lipids and does not exhibit the specific binding mechanism which the Cry proteins do (Höfte and Whiteley, 1989). Cyt proteins are thought to have a similar mode of action to that of Cry proteins, with the exception that they directly bind to the microvillar lipid bilayer. Similar to Bti, the toxicity of Bs is due to protein endotoxins produced during sporulation and assembled into a parasporal body. The main toxin of Bs is a binary toxin (BinA and BinB) which are proteolytically activated in the mosquito midgut to release peptides (43 and 39 kDa, respectively) to form the binary toxin. The toxins bind to microvilli of the midgut epithelium, trigger hypertrophy and lysis of cells, leading to destruction of midgut and death of mosquito larva.

3.2.3 Resistance

Despite decades of use, there is no reported resistance among mosquito populations probably due to biochemical properties of Cyt protein. Bti produces a parasporal body that contains four major endotoxins, Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa. Both Cry and Cyt are must be ingested to yield active toxins in the midget of insect. Cyt protein synergizes the toxicity of the Bti Cry proteins resulting in delayed the phenotypic expression of the evolution of resistance. Such synergistic interactions are also known to occur between the Bti Cry proteins. When Cyt protein combined with the Bs Bin toxin, it can also overcome resistance to Bti. For detailed information, see Wirth et al. (2005) and Federici (2010). As a rule, bacterial control agents are less likely to provoke resistance because their mode of action is more complex (Wirth et al., 2005; Federici, 2010). However, resistance had developed against Btk in some pests, e.x., the stored grain pest, *Plodia interpunctella*, and the diamondback moth, *Plutella xylostella*. In most cases, resistance has been associated with a recessive or partially recessive trait(s) and appears to be linked to a single gene. The combination of protoxins in Bti is very important in reducing the likelihood of resistance. In contrary, when the gene encoding a single toxin protein was cloned into a microorganism and then fed to larval mosquitoes, resistance was induced within a few generations; see transgenic organisms below and Mehlhorn (2011) for more details. For avoidance of resistance, products with different arrays of endotoxins should be alternated (Schuster & Stansly, 2006).

3.2.4 Safety

The U.S. Environmental Protection Agency (USEPA) categorizes the risk posed by Bt strains to nontarget organisms as minimal to nonexistent. After using of Bt products for more than 50 years, it can be concluded that Bt belong to the most environmentally safe products as they kill target organisms and usually do not harm nontarget organisms, such as beneficial insects, plants, or humans. The safety record for occupational exposure to Bt based biopesticides with regard to human health is considered good (Barfod, 2010). Some Bt

subspecies produce other types of toxins besides Cry proteins as ß-exotoxin and α-toxin. Both toxins are toxic to vertebrates and they are banned in Bt products, see Lacey & Kaya (2007) and Makonde et al. (2010) for more particulars about bacterial toxins. Bt and BS are the most used biocontrol agents against nuisance, pest or vector species. **T**hey are strongly incorporated in IPM programs because they are highly efficient, easily be mass-produced, easy to handle, stable when stored, cost-effective, pest specific, and safe to people and the environment. For more information about bacteria as a biological control agent, see Lacey & Kaya (2007), Gilbert & Gill (2010), Ahmed et al. (20 11), Mehlhorn (2011), Singh et al. (2011), and van Emden & Service (2011).

3.3 Entomophathogenic fungi

Entomophathogenic fungi are important natural control agents that limit insect populations. Survival of fungi requires a delicat balance of interactions between fungi, host, and the environment. Most fungi cause insect diseases spread by means of asexual spores called conidia which vary greatly in their ability to survive adverse environmental conditions, desiccation, and ultraviolet radiation.

3.3.1 Life cycle

In general, insects get infected when they come into contact with spores on the bodies of dead insects, on the surface of plants, in the soil, or in the air as windborne particles. High humidity is usually required for germination of conidia on the insect cuticle and production of germ tube that allow the fungus to penetrate the cuticle often at joints or creases where the insect's protective covering is thinner. In contrast to virus and bacteria, fungi do not have to be ingested to cause infections. The fungus multiplies within the host insect and kill it. Many fungi produce toxins to increase the speed of kill or prevent competition from other microbes. Death is due to toxin produced by the fungus or fungal multiplication. Under favorable conditions, the fungus grows out of the cadavers, usually at thinner areas and form conidiophores or analogous structures and sporulates. Some species go into a resting stage which survive periods of adverse conditions before forming or releasing spores. Soil incorporating fungi usually avoid the adverse effects of ultraviolet radiation and desiccation, but other microorganisms that act as competitors or antagonists often alter pathogen effectiveness. Fungal pathogens differ in the range of hosts. Many important fungi attack eggs, immature, and adults of a variety of insect species. Others are more specific to immature stages or to a narrow range of insect species. Several fungi are used to control insects such as *Leptolegnia spp., Coelomomyces spp., Hirsutella thompsonii, Nomuraea rileyi,* and *Vericillium lecanii*. The most famous entomophathogenic fungi will be disused below.

3.3.2 Oomycetes (water molds)

Lagenidium giganteum was first described by Couch (1935) from a combined collection of copepods and mosquito larvae, *Culex* and *Anopheles,* in North Carolina, USA. It has a wide geographical distribution: North America, Europe, Africa, Asia, Antarctica (Federici, 1981). The only species of the genus *Lagenidium* is known to be pathogenic to mosquito larvae is *L. giganteum (*formerly*: L. culicidum)*. As a facultative parasite, *L.giganteum* can grow as pathogen on mosquito larvae, or as a saprophyte in aquatic environments (Sur et al., 2001). This aquatic fungus is highly infectious to larvae of several mosquito genera. It cycles effectively in the aquatic environment even when mosquito density is low, but its

effectiveness is limited by high temperatures. The fungus is not effective for mosquitoes in brackish or organically rich aquatic habitats (Merriam and Axtell, 1982). *L. giganteum* has a wide geographical distribution: North America, Europe, Africa, Asia, Antarctica (Federici, 1981). Fungal reproduction is both asexual (zoospores) and sexual (oospores) (Federici, 1981). In order to infect mosquito larvae, biflagellate zoospores must be formed. Motile zoospores are the asexual stage of the fungus. Oospores, the sexual stage of *L.giganteum*, can also be used as inoculum. They are dormant propagules, resistant to desiccation and mechanical abrasion and stable for at least seven years, which allows multivoltine persistence of the fungus in some habitats (Kerwin et al., 1994).
Lagenidium spp. was isolated form Egypt for the first time form *Culex pipiens* larvae infesting a polluted creak in Miet El- Attar, Benha, Egypt, by Khater (2003). Such fungus was propagated on SDA medium and Peptone yeast glucose (PYG) for sporulation. Five concentrations (5 $x10^5$-1.6 $x10^7$) were used to infect *C. pipiens* late 3rd larval stage *In vitro* and the biological parameters had been followed up till emergence of adults. LC50 and LC90 values were 2.79 $x10^6$ and 3.94 $x10^8$ spores/ ml, respectively. At the lowest concentration, larval duration reached 8.3 days (3.6 days in the control group) and morphological changes (symptoms of mycosis) was 66.7%, (Fig. 4,5). All survived larvae died as pupae; consequently, the isolated fungus inhibited adult emergence. As this fungus has the ability to be self- propagated, it could be an effective control agent for the vector of Bancroftian filariasis and Rift valley fever virus in Egypt (Khater, 2003). The fungus has caused high mortalities in mosquito populations, see Schotle et al. (2004) for a review. Results from a small scale field trial in North Carolina indicated that *L. giganteum* recycled for an entire season despite periodic scarcity of hosts and short-term drought with infections ranging from 0-100% (Jaronski & Axtell, 1983). A large-scale field trial in Californian rice fields, using mycelium from either 20 or 30 liters of fermentation beer per hectare resulted in 40%-90% infection of *Culex tarsalis* and *Anopheles freeborni* sentinel larvae (Kerwin & Washino, 1987).

3.3.2.1 The relative potency

The relative potency indicated that Vectobac (Bti), and *Lagenidium* spp. were $12x10^{11}$ and 4.3 times, respectively, more effective than the fungal product Biosect®, *Beauveria bassiana*, on the basis of LC50 values (Khater, 2003). *L. giganteum* is compatible with the bacterial agents Bti and Bs Meyer and Neide when used against *Culex quinquefasciatus* (Orduz & Axtell, 1991), with the fungus having the distinct advantage over Bti in that it is able to recycle in stagnant water, infecting multiple and overlapping generations of mosquitoes (Legner, 1995). In field trials in which Laginex 25 was compared with Vectobac-12AS (Bti), Laginex reduced *C. quinquefasciatus* larvae by 100% for 22 days whereas Vectobac-12AS required retreatment by the 10th day (Hallmon et al., 2000).

3.3.2.2 Production

Unfortunately, mass production yields of oospores remain orders of magnitude below that of the less stable mycelial (asexual, presporangial) stage, and continued problems with spore activation have prevented large-scale field tests (Kerwin and Petersen, 1997). For solving this problem, improving oospore yields would be much more useful than zoospores in large-scale operational mosquito control programs. In contrast to the previous obstacles, Laginex® is a *L.giganteum*-based product by AgraQuest (California, USA) until 1999. It is effective against larvae of most pest mosquito species; remains infective in the environment through dry periods but it is unable to survive high summertime temperatures; the kind of

spore used was not mentioned. Shathele (2009) made an excellent potential for investigations on the use of newly developed isolate (Saudi-1 and Saudi-2) media for an isolated strain of *L. giganteum* form Louisiana for its maintenance and zoospore release.

3.3.2.3. Safety

Zoospores of the fungus appear harmless to vertebrates (Kerwin et al., 1990), most aquatic invertebrates (Nestrud & Anderson 1994) except *Daphnia* spp. and copepods (Couch, 1935), three cladoceran species and a chironomid species (Nestrud & Anderson, 1994).

3.3.3 Hyphomycetes

Most soil fungi used for the control of insect pests belong to the group hyphomycetes, such as *Beauveria bassiana* and *Metarhizium anisopliae,* formerly known as *Entomophthora anisopliae* . Some species have been developed as commercial products because of their ability to be mass produced. Most fungi in this group can cause natural outbreaks, to a wide range of insect hosts, on their own when environmental conditions are favorable. Soil provides protection against UV along with optimal conditions of temperature and moisture. Furthermore, the fungi may survive in the soil through recycling in insects or roots (Leger, 2008). Thus, they provide a long-term strategy for larvae and puparia control (Quesada-Moraga et al., 2006). *B. bassiana* infects both larvae and adults of a broad host range. Understanding the interactions between *B. bassiana* and other soil microorganisms is very important for the success of using of this fungus. Commertially available products based on *B. bassiana* are Mycotrol O (Emerald BioAgriculture), Naturalis Home and Garden (H&G), Naturalis L (Troy BioSciences, Inc.), and Biosect® (Kafr El Zayat - KZ Chemicals, Egypt). Khater (2003) used Biosect® to control larvae of both *M. domestica* and *C.pepiens In vitro*. LC50 an LC90 values were 29.2 $x10^7$ and 9.97 $x10^8$ spores/ ml for house flies and 1.2$x10^7$ and 4.17$x10^9$ spores/ ml, for mosquitoes, respectively. The total larval mortalities of mosquitoes were almost 100%, the few survived larvae died as pupae. At the lowest concentrations, Larval and pupal malformations were 30% and 45%, See fig (1,2,4,5) for symptoms of mycosis.

3.3.4 Entomophthorales

Entomophthorales is a group of fungi that tend to be much more host specific and can cause natural outbreaks in insect populations. Several different *Entomophthora muscae sensu stricto* genotypes were documented and each type was restricted to a single host species, indicating a very high degree of host specificity at or below the level of the subfamily **(**Jensen et al., 2001)**.** Currently, no commercially-based product is available because of difficulties in mass production. All available literature deal with *E. muscae* as a pathogenic fungi of adult *M. domestica,* but Khater (2003) isolated it for the first time in Egypt, form Moshtohor, Toukh, Qlubia governorate, such strain has a unique ability to infect larvae of house flies for the first time also. The isolated fungus was replicated on Sabouraud Dextrose Agar (SDA) medium for hyphenation and on liquid medium for laboratory – scale Entomophthora' hyphal production for enhancement of zoosporogenesis. Khater (2003) also made a bioassay using early 3rd larval stages of house flies which were infected *In vitro* by adding different concentrations of *E. muscae* (from 25 to 16 x 10^2 spores/ml) to the breeding medium of larvae and followrd up till adult emergence. LC_{50} and LC_{90} values for larval mortalities were 2.19 x 10^2 and 20 x 10^2 spores/ ml, respectively. At lower concentrations, larval durations were elongated (7.2 days) than that of the control group (2.6 days). Larval abnormalities were larval pigmentation, black, red- pin point pigmentation, ulceration, maceration and larval

pupal intermediates. Mortality and morphological changes of pupae (symptoms of mycosis) reached 100% at 8 x 10^2 spores /ml. Adult mortalities, deformity, and emergence reduction rates increased as the concentrations increased (79.3%, 60%, 98.2%, respectively, at 4x10^2 spores/ ml). Adult abnormalities were failure of adult eclosion, small adults, and deformed wings and legs (Fig. 1-3). In addition, this pathogen manipulates the behavior of infected host for his own dissemination as the apparently normal adults exhibited the characteristic position of *Entomophthora* spp. (Fig. 6) (Krasnoff et al., 1995; and Khater, 2003) as follows:

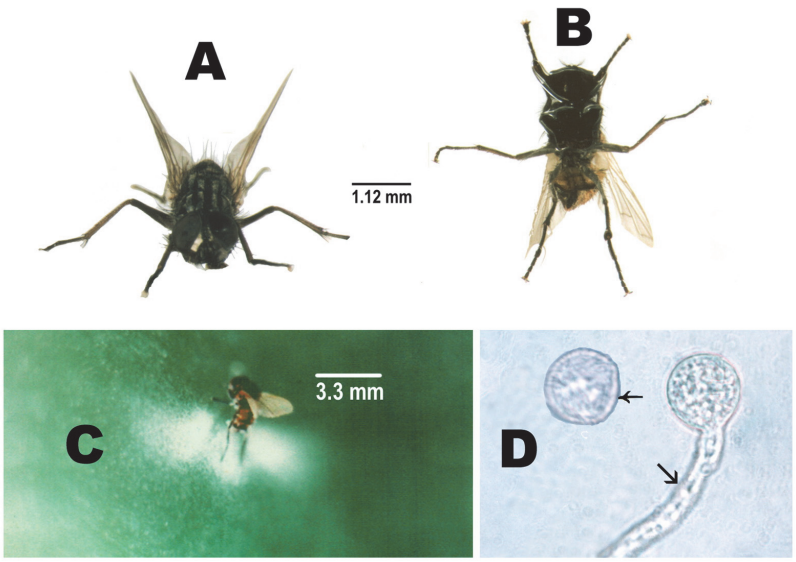


Fig. 6. Characters of adult house fly infected with *Entomophthora* spp.: A. Adult exhibited elevated abdomen, wings, and extended legs whereas the mouth parts were stoked downwards (anterior view), B. Adult with extended legs (ventral view), C. Conidial shower (white color), D. Hyphal body (zoosporangia) right arrow, spore (left arrow) of *Entomophthora* spp. Adapted form Khater (2003).

First, adults died in elevated positions, with the proboscis extended and attached to the substrate, legs spread, the abdomen angled away form the substrate, and the wings rose above the thorax. The conidial discharge reached 3-9 cm, depending on the size of the cadavers (Fig. 6). Conidia are forcibly ejected from conidiophores that emerge most profusely from the intersegmental membranes on the insect's abdomen, such posture enhance dispersal of conidia (Krasnoff et al., 1995; Khater, 2003). Similarly, an onion maggot fly, *Delia antiqua* (Meigen), infected by *E.muscae* Cohn (Fresenius) and dying at the top of a grass stem with its proboscis fastened to the substrate and its abdomen well raised by an atypical disposition of the legs appears to be an ideal launch pad for maximizing the effective area covered by the conidial shower (see Krasnoff et al., 1995 for more details). Second, the pathogen also benefits indirectly from the sexual attractiveness of female cadavers. Males attempting necrophilous copulations invariably become infected with the

fungus (Moller, 1993). Third, both field observations (Roffey, 1968) and controlled experiments (Bellini et al., 1992; Khater 2003, unpublished data) indicate that host mortality usually occurs in the late afternoon. This may be a temporal manipulation serving to enhance the likelihood that conidia produced from the dead host will be released at a time when the possibility for germination is highest, after dark when dew has set and surface moisture has had a chance to accumulate. The presumed adaptive payoff of elevation and posture for the fungus enhances dispersal of propagules.

3.3.5 Safety

Concerning the safety of entomopathogenic fungi developed for commercial use in microbial control of insect pests. Most fungi showed no infectivity to man or other vertebrates. Safety tests with *Nomuraea rileyi* , *Hirsutella thompsonii* , *Verticillium lecnii,* and *L. giganteum* assured negative findings to different mammals and birds. On the other hand, *B. bassiana* has been reported to cause allergies in humans and is at least an opportunistic pathogen to man and other mammals. *B. bassiana* and *M. anisopliae* affect non-target invertebrates with various degrees; see El-Husseini (2006) for more details. Soil treatment with *M. anisopliae* and *B. bassiana* on *Tapinoma nigerrimum* colonies indicated that there were no significant differences in ant, as a non- target host, activity before and after fungal treatment (Garrido-Jurado et al., 2011). For more information about entomophathogenic fungi, see Hakjek et al. (2007), Lacey & Kaya (2007), Gilbert & Gill (2010), Singh et al. (2011), and van Emden & Service (2001). For using of fungi for controlling arthropods of medical and veterinary importance, see Steenberg et al. (2001), Samuels, et al. (2002), Khater (2003), Kirkland et al. (2004), Schotle et al. (2004), Hartelt et al. (2008), Zabalgogeazcoa et al. (2008), Mochi et al. (2010), and Stephen & Kurtböke (2011).

3.4 Nematodes

Entomopathogenic nematodes cause damage to soilborne insect pests. Nematodes from the families Steinernematidae and Heterorhabditidae have proven to be the most effective as biological control organisms (Lacy & Kaya, 2007) to control a wide range of insect pests including filth flies, German cockroaches, cat fleas, armyworms, carpenter worms, crown borers, cutworms, flea beetles, leaf miners, mole crickets, phorid flies, plume moths, root weevils, sciarid flies, stem borers, webworms, and white grubs (Smart, 1995).

3.4.1 Life cycle

Generally speaking, the life cycle of most nematodes includes an egg stage, four juvenile stages, and an adult stage. The third juvenile stage, dauer, is the only infective and free-living stage which is capable of surviving in the soil; its function is to locate, attack, and infect an insect host through its breathing holes, mouth, or anus, but some species are capable of penetrating thin areas of the insect's cuticle. After that, the nematodes release special bacteria into the insect. The toxins produced by the bacteria kill the insect after a few days. The bacteria multiply inside the body of the insect and the nematodes eat the bacteria. The nematodes mature, mate, and multiply inside the insect. Eventually, the insect's body becomes filled with nematodes. Infective stage nematodes then exit the insect body searching of other insects to infect. Once inside the body cavity of the host, the infective

juveniles release bacteria that live symbiotically within the nematode's gut. The nematode-bacterium relationship is highly specific: only *Xenorhabdus* spp. bacteria co-exist with steinermatids, and only *Photorhabdus* bacteria co-exist with heterorhabditids. Under optimal conditions, it takes 3–7 days for steinernemadtids and heterorhabditids to complete one life cycle inside a host from egg to egg. Emergence of infective juveniles from the host requires about 6–11days for steinernemadtids and 12–14 days for heterorhabditids (Kaya & Koppenhöfer, 1999). The ability of any biological control nematode to infect a particular insect can be affected by nematode and insect behavior, physical barriers, and immune responses.

3.4.2 Using beneficial nematodes

Over 30 species of beneficial nematodes have been identified. Seven species have been commercialized worldwide and seven are currently available in the United States: *Steinernema carpocapsae, S. feltiae, S. glaseri, S. riobravis, Heterorhabditis bacteriophora, H. megidis,* and *H. marelatus.* (Kaya & Koppenhöfer, 1999). There were two commercial nematode products available for termite control, Spear ® and Saf T-Shield ®.

The use of the entomopathogenic nematodes *S. feltiae* and *H. megidis* in high numbers was shown to be effective against housefly larvae (Renn, 1995) and effective after formulation into a housefly bait, as effective as the spraying of a carbamate pesticide in pig units (Renn, 1998). Also the nematode *Paraiotonchium muscadomesticae* could infect housefly larvae, but mortality was low except at high nematode concentrations, but *P. muscadomesticae* infect housefly larvae and descendants of the nematodes invade and damage the ovaries of adult female flies and are deposited in the larval habitat when the flies attempt to oviposit. Infected adults lived about half as long as uninfected flies (Geden, 1997; Khater, unbuplished data); consequently, *P. muscadomesticae* reduce fly population indirectly. Nematodes were effective in the laboratory but persisted in manure only for 3-7 days. Moreover, mermithid nematodes which parasitize grasshoppers, cockroaches, and mosquitoes can play a part in natural suppression of pest insects. Nematodes must be raised in live insects and this is not a very efficient means of production.

3.4.3 Safety

Entomopathogenic nematodes are not harmful to humans, animals, plants, or earthworms. They can therefore be used as biological control organisms. Such beneficial nematodes have been released extensively in the field with negligible effects on nontarget insects and are regarded as remarkably safe to the environment.

Finally, microbial control agents provide a more environmentally acceptable and sustainable form of insect pest management than chemical insecticides, but are most effective when underpinned by a detailed knowledge of specific host-pathogen interactions. For more information about successful application of entompathgenic nematodes, see Mill et al. (2000), Gaugler (2002), Crow (2006), Hajek et al. (2007), Lacey & Kaya (2007), and Gilbert & Gill (2010).

4. Transgenic pesticides

Genetically modified organisms (GMO) and crops (GMC) could be engineered to augment pest control. Transgenic organisms are genetically altered by artificial introduction of DNA

from another organism and the artificial gene sequence is referred to as a transgene. Plants with such transgenes are called GMC, plant pesticides or plant incorporated protectants.

4.1 Genetically modified crops

By the mid 1980s, one company, Monsanto had committed to a research program designed to create crop protection products through the application of biotechnology (Glover, 2008). Charles (2001) has produced a very readable history of pesticide-related transgenic crops. Different endotoxins have different biological spectra, thus different genes are used in GMC, for protection from attack by various insects. For example, Monsanto has used *cryIA(c)* genes from Btk in cotton and tomatoes and *cryIIIA* genes from Btt in potatoes. Moreover, Novartis and Mycogen used *cryIA(b)* genes from Btk and Monsanto used *cryIIA* and *cryIA(b)* genes, both from Btk in corn. AgrEvo (Plant Genetic Systems) is using *cry9C* toxin genes from *B thuringiensis* subsp *tolworthi* in transgenic corn. GMC have been enhanced sometimes by use of stacked genes. i.e. more than one transgene is introduced into the same crop to achieve multiple desired characteristics and avoidance of resistance. Many experimental studies conducted to date indicate that transgenic plants have no adverse effects on non-target organisms. In addition, there is no scientific evidence as yet that the commercial cultivation of GM crops has caused environmental impacts beyond the impacts that have been caused by conventional agricultural management practices. Nonetheless, studies are still on-going to assess the potential environmental impacts of GM crops (see Makonde et al., 2010). The effect of GM crops on natural enemies remains a controversial topic (Andow et al., 2006; Marvier et al., 2007; Lövei et al., 2009).

4.2 Genetically modified organisms

Molecular biology impact searching for new products through understanding the chemistry and mode of action of a natural product and the discovery of a new protein (and its gene) that may be used to transform a target crop or organism. For more information about proteases as insecticidal agents; see Harrison & Bonning (2010).

4.2.1 Recombinant bacteria

It has been shown that plasmids with a molecular weight of 60–94 MDa play an essential role in the crystal toxin production. Cloning various toxin genes into host organism's increase the persistence or the insecticidal properties. The development of recombinant DNA techniques improves the efficacy of Bti through combining the most potent insecticidal proteins from Bti, Btj, and Bs into new bacterial strains that are ten-fold more toxic than wild type species of Bti and Bs used in current commercial formulations. New bacterial larvicides offer environmentally compatible options for use as components in integrated vector control programs aimed at reducing malaria, filariasis, and many important viral diseases of humans (Federici, 2010). Recombinant (new) bacterial larvicides are used as components in IPM programs because they are much more highly efficient than the wild type strains from which they were originally derived. Their costs are similar to that of new chemical insecticides, and they are much more environmentally compatible than most chemical insecticides. Therefore, recombinant bacterial larvicides will play an important role in controlling pests and vectors in the near future. For more information, see Berón & Salerno (2007), Lacey & Kaya (2007), Gilbert & Gill (2010), Federici (2010), and Singh et al. (2011).

4.2.2 Recombinant baculoviruses

Most insect viruses take several days to kill their host insect. In the same time, the pest is still causing damage. Very high doses are often necessary for adequate control and early larval life stages are highly most susceptible to virus infection. For solving the previous problems, there has been steady interest in the potential to produce recombinant BVs to increase their speed of kill or reduce insect feeding (Inceoglu et al., 2001), The activity of BVs against their natural hosts may be enhanced by introduction of insect-specific toxins, such as toxin genes isolated from the scorpion or spiders, or by interference with insect physiology (Bonning & Hammock, 1996). Arthropod toxins usually attack insect sodium channels producing final effect similar to the chemical insecticides of the pyrethroid group. Though, the specific target in sodium channels is different, so there is a potential possibility to produce synergistic effect by biopesticide/chemical pesticide application (McCutchen et al., 1997). BV recombinants that produced occlusion bodies incorporating Bt toxin were constructed by making a fusion protein consisting of polyhedron and Bt toxin (Chang et al., 2003). This new biopesticide is highly pathogenic than wild type virus as it combines the advantages of the virus and the bacterial toxin.

The changes to host physiology were done by introducing genes coding for some insect hormones or hormone-modifying enzymes into BV genome, or by deletion of the BV-encoded ecdysteroid glucosyltransferase (*egt*) gene. Cloning juvenile hormone esterase gene into BV genome which over expressed decreases the concentration of the juvenile hormone which is a signal for a caterpillar to stop feeding and pupate (Inceoglu et al., 2001). The product of the *egt* gene interacts with larval moulting and indirectly affect the time of feeding of infected caterpillars. Furthermore, recombinant juvenile hormone esterase act as a biochemical anti- JH hormone agent and it affects ovarian development in the house cricket *Acheta domesticu (*Bonning et al., 1997). The deletion of the baculovirus encoded *egt* gene was used first by O'Reilly and Miller (1991). Such deletion from BV genome resulted in 30% faster killing of caterpillars. The *egt* gene is not essential for viral replication and can be replaced with an exogenous gene; the product of which may enhance the insecticidal activity of the recombinant virus (Sun et al., 2004). Genetically modified BVs are safe as they infect only their hosts. They are not pathogenic to bees and vertebrate species (Sun et al., 2004) as well as to the natural enemies of larvae such as parasitoids and predators (Boughton et al., 2003). Consequently, recombinant BVs lead to the expansion of BV use worldwide. For more details about recombinant BVs, see Szewczyk et al. (2009, 2011), Gilbert & Gill (2010), Ahmed et al. (2011), and Singh et al. (2011).

4.2.3 Transgenic insects

Genetic modification of mosquitoes (which renders them genetically modified organisms, GMOs) offers opportunities for controlling malaria. Transgenic strains of mosquitoes have been developed to replace or suppress wild vector populations and reduce transmission and deliver public health gains. The transition of this approach from confined laboratory settings to open field trials in disease endemic countries (DECs) is a staged process that aims to maximize the likelihood of epidemiologic benefits while minimizing potential pitfalls during implementation. Unlike conventional approaches to vector control, application of GM mosquitoes will face contrasting expectations of multiple stakeholders, the management of which will prove critical to safeguard support and avoid antagonism, so that potential public health benefits can be fully evaluated. Inclusion of key stakeholders in decision-

making processes, transfer of problem-ownership to DECs, and increased support from the wider malaria research community are important prerequisites for this. It is argued that the many developments in this field require coordination by an international entity to serve as a guiding coalition to stimulate collaborative research and facilitate stakeholder involvement. Contemporary developments in the field of modern biotechnology, and in particular GM, requires competencies beyond the field of biology, and the future of transgenic mosquitoes will hinge on the ability to govern the process of their introduction in societies in which perceived risks may outweigh rational and responsible involvement (Knols et al., 2007). For more information about of transgenic insects and improving their ecological safety for field release, see Vreysen et al. (2007).

5. Future trends, nanoparticles

The potential uses and benefits of nanotechnology are enormous. These include enhancement involving nanocapsules for vector and pest management and nanosensors for pest detection. Nanoparticles are 1-100 nm in diameter, whereas the size of a virus is roughly 100 nm. Such particles are agglomerated atom by atom. Nanotechnology deals with the targeted nanoparticles which exhibit different physical strength, chemical reactivity, electrical conductance, and magnetic properties. Nanoparticles are present in insect entire body parts. Insects are incredible nanotechnologists. The surfaces of many insect wings have evolved properties materials scientists only dream of for their creations (Watson et al., 2010). Nanoparticles help to produce new pesticides, insecticides and insect repellants (Owolade et al., 2008). Nanoencapsulation is a process through which a chemical (ex. an insecticide) is slowly but efficiently released to a particular host for insect pest control. Release mechanisms include diffusion, dissolution, biodegradation and osmotic pressure with specific pH (Vidhyalakshmi et al., 2009). Such process can also deliver DNA and other desired chemicals into plant tissues for protection of host plants against insect pests (Torney, 2009). Nanoparticles loaded with garlic essential oil are efficacious against *Tribolium castaneum* Herbst *(*Yang et al., 2009). Aluminosilicate filled nanotube can stick to plant surfaces while nano ingredients of nanotube have the ability to stick to the surface hair of insect pests and ultimately enters the body and influences certain physiological functions (Patil, 2009).

Nanotechnology is used widely in agriculture and food (Joseph & Morrison, 2006). One of the world's largest agrochemical corporations, Syngenta, is using nanoemulsions in its pesticide products. Encapsulated product from Syngenta delivers a broad control spectrum on primary and secondary insect pests of cotton, rice, peanuts, and soybeans. Marketed under the name Karate® ZEON, a quick release microencapsulated product containing the active compound lambda-cyhalothrin (a synthetic insecticide based on the structure of natural pyrethrins) which breaks open on contact with leaves. The encapsulated product "gutbuster" only breaks open to release its contents when it comes into contact with alkaline environments, such as the stomach of certain insects. Furthermore, the new technology improve pesticide and fertilizer delivery systems which can take action to environmental changes, ex. they will release their cargo in a controlled manner (slowly or quickly) in response to different signals e.g. heat, moisture, ultrasound, magnetic fields, etc. Recently, nanotechnology is widely acceptable publicly because it is not yet linked to any toxicological and ecotoxicological risks. Research on nanoparticles and insect control should be directed toward production of faster and ecofriendly pesticides to deliver into the target host tissue

through nanoencapsulation. This will control pests efficiently and accelerate the green revolution. For more in rank about usages of nanoparticles, see Joseph & Morrison (2006), Torney (2009), Yang et al. (2009), Bhattacharyya et al. (2008, 2010), Ahmed et al. (2011), and Hashim (2011).

6. Conclusion

IPM programmes have demonstrated that current levels of pesticide use in many circumstances are not necessary and, frequently, are even counter-productive. Excessive and otherwise inappropriate pesticide use is an unnecessary burden on farmers' health and income, on public health, and on the environment (Pretty, 2009). Biorational insecticides have emerged as an alternative or as supplemental forms of pest control. The use of biopesticides will help in preventing the discarding of thousands of tons of pesticides on the earth and provide the residue free food and a safe environment to live. In spite of intensive research on plant natural products and insect-plant chemical interactions over the past three decades, only two new types of botanical insecticides have been commercialized in the past 15 years, those based on neem seed extracts (azadirachtin), and those based on plant essential oils. There are some obstacles toward commercialization of new botanical products, such as the availability of sufficient quantities of plant material to produce the pesticides, standardization, refinement, and quality control of the products; regulatory approval, patents, difficulties in registration; as well as problems related to their volatility, poor water solubility, and aptitude for oxidation. These challenges should be overcome for botanicals to be of particular use for human and high value animals and crops. For more information about standardization, regulatory approval, and commercialization of botanical insecticides, see Tripathi et al. (2009), Isman (2006, 2010), Dubey (2011), and Isman et al. (2011). The insect growth regulator, nonsteroidal ecdysone agonist bisacylhydrazine (BSH) insecticides are generally faster acting than the JHA and CSI insecticides, thus, preventing crop damage by inhibition of feeding within 3–12 h after application. Although both JHA and CSI were discovered long before the BSH, the mode of action of BSH is the best understood at the molecular level. This allows cloning and expression of cDNAs encoding the ecdysone receptor complex, a heterodimer of two proteins: ecdysone receptor (EcR) and ultraspiracle (USP), from several insects, and the availability of stable and easy to synthesize BSH. Moreover, the molecular targets for BSH and reasons for the selective insect toxicity of bisacylhydrazine insecticides are also well known and understood. More investigations are required to explore several aspects about other IGRs, for instance, the molecular targets and basis of actions of about JHAs and CSI and if the JHAs use the same molecular target/site as the natural JHs do. Development of more efficient high-throughput assays for discovery of new and novel CSIs is allowed through understanding of the biosynthetic pathway for chitin synthesis and cuticle deposition, molecular characterization of the various enzymes involved, and the precise mode of action of CSIs, see Gilbert & Gill (2010) for more details. For designing better applications and protocols for pheromone application, understanding the mechanisms of mating disruption systems for different target species and dispensers is very curtail. It is better to suppress highly mobile insects at regional rather than local scales and use newer formulations, such as puffers, attract-and-kill, and sprayable formulations, that offer opportunities to reduce costs, increase program flexibility, and mix strategies. More reliable, economic, and widespread applications of pheromone can be achieved if a joint effort is made by people from different disciplines and organizations. Because of some

difficulties with high populations of pests, pheromones should not be viewed as stand-alone strategies but integrated with other IPM options.

Simply, biological control could be defined as using biota to reduce biota safely and economically. In the last 50 years, microbial insecticides illustrate remarkable developments associated with distinct good results under optimized laboratory conditions, followed many times by unsatisfactory results in the field applications. It is very important to produce reliable, effective, and safe entomo-pathogens for microbial control through the integration of research efforts about physiology, pathology, genetics, mass production, formulation, and application strategies, see Ravensberg (2011), for more information about a roadmap to the successful development and commercialization of microbial pest control products for control of arthropods. Production of biopesticides sharpens the action of genetic engineering for amalgamation of two or more effective lethal processes to finally tailor them into one agent/organism. As a result, the probability of development of resistant strains will decrease due to little morbidity and effective mortality. In spite of concerns about the potential environmental hazards of their long-term use, such opportunities will continue to persuade companies to seek new products and producers. GM microbes and transgenic crops are the new comer in IPM strategies and are gaining popularity because of their efficacy in eradicating pests. Nanotechnology is a promising field of research launches in the present decade a wide array of prospects and is anticipated to give major force in pest control. It is accepted by the public and not yet linked to any great concerns about health and the environment.

Limited shelf-life, high specificity and variable field performance could be considered as advantages and disadvantages of biorational insecticides. As a result, proper identification of a target insect pest is essential and biorational products must be applied when the pest is in its most vulnerable life stage. Otherwise, applications may be ineffective, and applications of a conventional product may be necessary. Insect could also be controlled through environmental/ cultural controls as well as genetic control, see Vreysen et al. (2007), Gurr & Kvedaras (2010), Atkinson (2010), and van Emden & Service (2011) for more details. For more information abut area-wide control of insect pests from research to field implementation, see Vreysen et al. (2007). Achieving a zero pesticide strategy in tropical agroecosystems may be easier than in temperate zones, as in many instances farmers have not yet begun the generalized use of pesticides. This gives special opportunities for scientists and farmers to work on a systems approach to minimize pest impact before agroecosystems have been disturbed. For those areas already heavily impacted by the use of pesticides, such as cash crops, horticultural crops and livestock, adjustment to pesticide-free systems management will take some adaptation. Time is needed to establish or re-establish conditions in the system that are conducive to increased natural control such as habitat management and agronomic practices,.as well as to introduce farmers to new biological and physical control concepts and methods (Pretty, 2009). In general, biorational insecticides are much slower acting than those acting on neural target sites. The end user has, of course, been used to seeing insects die within a very short time following application of neuroactive insecticides. The change to the new insecticides has necessitated educating the distributors and the users on the mode of action and safety of the new products. There is a great potential to clean up agriculture form conventional insecticides. The road will be open to detoxify agriculture in case of endorsement of strong political will, consumer awareness, and market responses. Indisputably, the number and quality of biorational products will

increase and the costs will fall. These progresses will assure an increasing place in the market for biopesticides for the foreseeable future.

7. Acknowledgements

The author would like to express her sincerest gratitude to Prof. Dr. Aza Moustafa, Professor of Insecticides at the Research Institute of Medical Entomology, Egypt) for her support and suggestions; Prof. Dr. Adel Shaheen, Head of the Department of Fish Diseases and management, Faculty of Veterinary Medicine, Benha University, Egypt and the father of aquatic mycotic diseases in Egypt, for his honest help and advise; Prof. Dr. Abdou Mahdi, Plant Pathology Department, Faculty of Agriculture, Benha University, Egypt, for his helpful advice about the mycotic part of this work; Prof. Dr. Mohamed Hafez, Plant Pathology Department, Faculty of Agriculture, Benha University, Egypt, for his valuable help with the viral part of this chapter as well as the quality of the figures; Dr. Nagwa Ahmed, Dr. Mohsen Negm El Din and and Lubna El- Akabawi, Department of Parasitology, Faculty of Veterinary Medicine, Benha University, Egypt, for their valuable advice.

8. References

Ahmad, I., Ahmad, F. & Pichtel, J. (2011). *Microbes and Microbial Technology,* Springer.

Andow, D. A., Lövei, G. L. & Arpaia, S. (2006). *Bt* transgenic crops, natural enemies and implications for environmental risk assessment, *Nature Biotechnology* 24: 749-751.

Atkinson, PW. (2010). *Vector Biology, Ecology and Control*, Springer. DOI 10.1007/978-90-481-2458-9

Awad, HH., El-Tayeb, TA., Abd El-Aziz, NM. & Abdelkader, MH. (2008). A semi-field study on the effect of novel hematoporphyrin formula on the control of *Culex pipiens* larvae, *Journal of Agriculture & Social Sciences* 4: 85–8

Barfod, KK. (2010). *Occupational exposure to Bacillus thuringiensis based biopesticides, Development of an aerosol hazard identification model.* University of Copenhagen, PhD Dissertation.

Baum, JA., Johnson, TB. & Carlton, BC. (1999). *Bacillus thuringiensis* natural and recombinant bioinsecticide products, in FR. Hall & JJ. Menn (eds), *biopesticides use and delivery*, Humana Press, Totowa, New Jersey, pp. 189–209.

Bellini, R., Mullens. B. A. & Jespersen. J.B. (1992). Infectivity of two members of the *Entomophthora muscae* complex (Zygomycetes: Entomophthorales) for *Musca domestica* (Diptera: Muscidae), *Entomophaga* 37: 1 l-19.

Ben Amor, T. & Jori, G. (2000). Sunlight-activated insecticides: historical background and mechanisms of phototoxic activity, *Insect Biochemistry and Molecular Biology* 30: 915–925

Berón, CM., & Salerno, GL. (2007). Cloning and Characterization of a Novel Crystal Protein from a Native *Bacillus thuringiensis* Isolate Highly Active Against *Aedes aegypti,* Current Microbiology 54: 271–276.

Betarbet, R., Sherer, TB., MacKenzie, G., Garcia-Osuna, M., Panov, AV. & Greenamyre, JT. (2000). Chronic systematic pesticide exposure reproduces features of Parkinson's diseas, *Nature Neuroscience* 3:1301–1306.

Bhattacharyya, A., Bhaumik, A., Rani, PU., Mandals. & Epidi, TT. (2010). Nano-particles - A recent approach to insect pest control, *African Journal of Biotechnology* 9 (24): 3489-3493.

Bhattacharyya, A., Gosh, M., Chinnaswamy, KP., Sen, P., Barik, B., Kundu, P. & Mandal, S. (2008). Nano-particle (allelochemicals) and Silkworm Physiology, in KP. Chinnaswamy & Rao A Vijaya Bhaskar (eds), *Recent Trends in Seribiotechnology*, Bangalore. Indian. pp. 58-63.

Boeke, SJ., Boersma, MG., Alink, GM., van Loon, JJA., van Huis A., Dicke, M. & Rietjens, IMCM. (2004). Safety evaluation of neem (*Azadirachta indica*) derived pesticides, *Journal of Ethnopharmacology* 94: 25–41.

Bomford, MK. & Isman, MB. (1996). Desensitization of fifth instar *Spodoptera litura* (Lepidoptera: Noctuidae) to azadirachtin and neem, *Entomologia Experimentalis et Applicata* 81:307–13.

Bonning, BC. & Hammock, B D. (1996). Development of recombinant baculoviruses for insect control. *Annual review of Entomology* 41:191-210.

Bonning, BC., Loher, W., Bruce, D. & Hammock, BD. (1997). Recombinant Juvenile Hormone Esterase as a Biochemical Anti-Juvenile Hormone Agent: Effects on Ovarian Development in *Acheta domesticus, Archives of Insect Biochemistry and Physiology* 34:359–368.

Boughton, AJ., Obrycki, JJ. & Bonning, BC. (2003). Effects of a protease-expressing recombinant baculovirus on nontarget insect predators of *Heliothis virescens*, *Biological Control*, 28: 101 –110.

Bowen, Fl., Fisara, P., Junquera P., Keevers, DT., Mahoney, RH. & Schmid, HR. (1999). Long-lasting prevention against blowfly strike using the insect growth regulator dicyclanil, *Australian Veterinary Journal*. 77 (7): 454–460.

Bowers, W.S. & Nishida, R. (1980). Juvocimences L potent juvenile hormone mimics from sweet basil, *Science* 209: 1030–1032.

Bray, DP., Bandi, KK., Brazil, RP., Oliveira, AG. & Hamilton, JG. (2009). Synthetic sex pheromone attracts the leishmaniasis vector *Lutzomyia longipalpis* (Diptera: Psychodidae) to traps in the field, Journal of Medical Entomology 46(3): 428-34.

Casida, JE. & Quistad, GB. (1995). *Pyrethrum Flowers: Production, Chemistry, Toxicology and Uses*. Oxford, UK: Oxford Univ.Press. 356 pp.

Chang, JH., Choi, JY., Jin, BR., Roh, JY., Olszewski, JA., Seo, SJ., et al., (2003). An improved baculovirus insecticide producing occlusion bodies that contain *Bacillus thuringiensis* insect toxin, *Journal of Invertebrate Pathology* 84:30-37.

Charles, D. (2001). *Lords of the harvest: Biotech, big money, and the future of food*. Perseus Publishing, Cambridge, MA, 348 pp.

Collins, D.A. (2006). A review of alternatives to organophosphorus compounds for the control of storage mites, *Journal of Stored Products Research* 42: 395–426

Copping, LG. & Menn, JJ. (2000). Biopesticides: a review of their action, applications and efficacy, *Pest Management Science* 56: 651- 76.

Couch, JN. (1935). A new saprofytic species of *Coelomomyces*, with notes on other forms, *Mycologia* 27: 376-387.

Cristofari-Marquand, Ec., Kacel, M., Milhe, F., Magnan, A. & Lehucher-Michel, M. (2007). Asthma caused by peracetic acid-hydrogen peroxide mixture, Occupatinal Health 49 (2): 155–158.

Crow, WT. (2006). Using Nematodes to Control Insects: Overview and Frequently Asked Questions. ENY-050 (IN468), one of a series of the Entomology & Nematology Department, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida. http://edis.ifas.ufl.edu/ TOPIC_GUIDE_Insect_Management_Guide.

De Groot, I.J.M. (1991). *Protection of Stored Grains and Pulses*, Agrodok 18 Agromisa Foundation, Wageningen, The Netherlands, 45 pp.

Dethier, V.G. (1982). Mechanisms of host plant recognition, *Entomologia Experimentalis et Applicata* 31: 49-56.

Dubey, N.K. (2011). *Natural Products in Plant Pest Management*, CAB International.

El-Husseini, M.M. (2006). Microbial Control of Insect Pests: is it an effective and environmentally safe alternative? *Arab Journal of Plant Protection* 24 (2): 162-169.

Enan, EE. (2005). Molecular and pharmacological analysis of an octopamine receptor from American cockroach and fruit fly in response to plant essential oils, *Archives of Insect Biochemistry and Physiology* 59: 161-171.

EPA. (1993). Flower and Vegetable Oils. Prevention, Pesticides, and Toxic Substances (7508W). EPA-738-F-93-027. www.epa.gov/oppsrrd1/REDs/factsheets/4097fact.pdf

Eto, M. (1990). Biochemical mechanism of insecticidal activities, in G. Haug & H. Hoffman (eds.) *Chemistry of Plant Protection*, Springer Verlag. 6: 65-107.

Federici, BA. (1981). Mosquito control by the Fungi *Culicinomyces Coelomomyces* and *Coelomomyces*. in HD. Burges. (ed.) *Microbial Control of Pests and Plant Diseases* 1970-1980, London, Academic Press, 29: 555-572.

Federici, BA. (2010). Recombinant bacterial larvicides for control of important mosquito vectors of disease, in PW. Atkinson (ed.), *Vector Biology, Ecology and Control*, Springer, Part III, 163-176.

Fillinger, U. & Lindsay, SW. (2000). Suppression of exposure to malaria vectors by an order of magnitude using microbial larvicides in rural Kenya, *Tropical Medicine and International Health* 11:1629–1642.

Garrido-Jurado, I., Ruano, F., Campos M. & Quesada-Moraga, E. (2011). Effects of soil treatments with entomopathogenic fungi on soil dwelling non-target arthropods at a commercial olive orchard. Biological Control, in Press.

Gaugler, R. (2002). *Entomopathogenic nematology,* CABI Publishing

Geden, C.J. (1997). Evaluation of *Paraiotonchium muscadomesticae* (Nematoda: Tylenchida: Iotonchiidae), a potential biological control agent of the housefly (Diptera: Muscidae), *Biological Control* 10: 42-47.

Gilbert, LI. & Gill, SS. (2010). *Insect Control: Biological and Synthetic Agents*. Academic Press.

Gill, S.A., Reeser, R. & Raupp, M.J. (1998). Battling thrips, five pesticides put to the test, *Grower Talks* 62: 46-48.

Glover, D. (2008). *The Corporate Shaping of GM Crops as a Technology for the Poor*, Made by Monsanto, www.steps-centre.org/PDFs/GM%20Crops%20web%20final_small.pdf

Graf, JF. (1993).The role of insect growth regulators in arthropod control, Parasitology Today 9 (12): 471-474.

Gunasena, HPM. & Marambe, B. (1998). *Neem in Sri Lanka: A monograph*. University of Peradeniya, Sri Lanka.

Gurr, G.M. & Kvedaras, O.L. (2010). Synergizing biological control scope for sterile insect technique, induced plant defenses and cultural techniques to enhance natural enemy impact, Biological Control 52 (3): 198-207.

Hajek, AE., McManus, M.L.& Delalibera Junior, I. (2007). A review of introductions of pathogens and nematodes for classical biological control of insects and mites, *Biological Control* 41: 1-13.

Hallmon, CF., Schreiber, ET., Vo, T. & Bloomquist, MA. (2000). Field trials of three concentrations of Laginex as biological larvicide compared to Vectobac-12AS as a biocontrol agent for *Culex quinquefasciatus. Journal of the American Mosquito Control Association* 16: 5-8.

Harrison, L. & Bonning, BC. (2010). Proteases as Insecticidal Agents. *Toxins* 2: 935-953.

Hartelt, K., Wurst E., Collatz, J., Zimmermann, G., Kleespies, RG., Oehme, RM., Kimmig, P., Steidle, JLM. & Mackenstedt, U. (2008). Biological control of the tick *Ixodes ricinus* with entomopathogenic fungi and nematodes: Preliminary results from laboratory experiments, International Journal of Medical Microbiology 298 (1): 314-320.

Hashim, A. (2011). *Advances in nanocomposite technology,* InTech, Croatia.

Hohmann, CL., Flávia, A C., Silva, FAC. & de Novaes, TG. (2010). Selectivity of neem to *Trichogramma pretiosum* Riley and *Trichogrammatoidea annulata* De Santis (Hymenoptera: Trichogrammatidae), Neotropical Entomology 39(6):985-990.

Hollingworth, R.M., Johnstone, E.M. & Wright, N. (1984). In: P.S. Magee, G.K. Kohn and J.J. Menn (eds.), *Pesticide Synthesis through Rational Approaches, ACS Symposium Series No. 255*, American Chemical Society, Washington, DC (1984), pp. 103–125.

Hoover, K., Kishida, KT., DiGiorgio, L.A., Workman, J., Alaniz, S.A., Hammock, B.D. & Duffey, S.S. (1998). Inhibition of baculoviral disease by plant-mediated peroxidase activity and free radical generation. *Journal of Chemical Ecology* 24: 1949-2001.

Höfte, H. & Whiteley, HR. (1989). Insecticidal crystal proteins of *Bacillus thuringiensis. Microbiology and Molecular Biology Reviews* 53:242–255.

Inceoglu, AB., Kamita, SG., Hinton, AC., Huang, Q., Severson, TF.,Kang, KD. & Hammock, B. D. (2001). Recombinant baculoviruses for insect control. *Pest Management, Science* 57: 981 - 7.

Isman, MB. (2005). Problems and opportunities for the commercialization of botanical insecticides, in: C. Regnault-Roger, BJR. Philog` ene. & C. Vincent (eds.), *Biopesticides of Plant Origin,* pp. 283–91. Paris: Lavoisier.

Isman, M.B. (2006). Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world, *Annual Review of Entomology* 51:45–66.

Isman, MB. (2010). Botanical insecticides, deterrents, repellents and oils, in B.P. Singh (ed.), in: *Industrial crops and uses*. CAP International, pp. 433-445.

Isman, M.B. & Akhtar, Y. (2007). Plant natural products as a source for developing environmentally acceptable insecticides. in: I. Shaaya, R. Nauen, & A.R. Horowitz

(eds), *Insecticides Design Using Advanced Technologies.* Springer, Berlin, Heidelberg, pp. 235–248.

Isman, MB., Miresmailli, S. & Machial, C. (2011). Commercial opportunities for pesticides based on plant essential oils in agriculture, industry and consumer products. *Phytochemistry Reviews,* 10:197–204.

Isman, MB., Matsuura, H., MacKinnon, S., Durst, T., Towers, GHN. & Arnason, JT. (1996). Phytochemistry of the Meliaceae. So many terpenoids, so few insecticides. In JT. Romeo, JA. Saunders & P. Barbosa (eds.) *Phytochemical Diversity and Redundancy*, New York: Plenum, pp. 155– 78.

Jaronski, S. & Axtell, RC. (1983). Persistence of the mosquito fungal pathogen *Coelomomyces giganteum* (Oomycetes; Lagenidiales) after introduction into natural habitats, *Mosquito News* 43; 332-337.

Jehle, JA., Blissard, G.W., Bonning B.C., Cory, J.S, Herniou, E.A, Rohrmann, G.F., Theilmann, D.A., Thiem, S.A. & Vlak,. J.M. (2006). On the classification and nomenclature of baculoviruses: a proposal for revision. *Archives of Virology* 151: 1257–1266.

Jensen, AB., Thomsen L. & Eilenberg J. (2001). Intraspecific Variation and Host Specificity of *Entomophthora muscae sensu stricto* Isolates Revealed by Random Amplified Polymorphic DNA, Universal Primed PCR, PCR-Restriction Fragment Length Polymorphism, and Conidial Morphology, Journal of Invertebrate Pathology 78 (4): 251-259.

Joseph, T. & Morrison, M. (2006). *Nanotechnology in Agriculture and Food*. A Nanoforum report. www.nanoforum.org .

Karmakar, P.R. & Chatterjee, B.P. (1994). Isolation and characterization of two IgE-reactive proteins from *Azadirachta indica* pollen, *Molecular and Cellular Biochemistry* 131: 87–96.

Kaya, H. K. & Koppenhöfer, A.M. (1999). Biology and ecology of insecticidal nematodes, in S. Polavarapu (ed.) workshop proceedings: *Optimal use of insecticidal nematodes in pest management*, Rutgers University, p. 1–8.

Kerwin, JL. & Petersen, EE. (1997). Fungi: Oomycetes and Chytridiomycetes. In: Lacey LA. (ed.), Manual of techniques in insect pathology, 5-4: 251- 268. New York. Academic Press.

Kerwin, JL. & Washino, RK. (1987). Ground and aerial application of the asexual stage of *Coelomomyces giganteum* for control of mosquitoes associated with rice culture in the central valley of California, *Journal of the American Mosquito Control Association* 3: 59-64.

Kerwin, JL., Dritz, DA. & Washino, RK. (1990). Confirmation of the safety of *Coelomomyces giganteum* (Oomycetes: Lagenidiales) to mammals. *Journal of Economic Entomology* 83: 374-376.

Kerwin, JL., Dritz, DA. & Washino, RK. (1994). Pilot scale production and application in wildlife ponds of *Coelomomyces giganteum* (Oomycetes: Lagenidiales), *Journal of the American Mosquito Control Association* 10: 451-455.

Khater, H.F. & Khater, D.H. (2009). The insecticidal activity of four medicinal plants against the blowfly *Lucilia sericata* (Diptera: Calliphoridae), *International Journal of Dermatology* 48 (5): 492- 497.

Khater, H.F. & Ramadan, M.Y. (2007). The acaricidal effect of peracetic acid against *Boophilus annulatus* and *Argas persicus*, *Acta Scientiae Veterinariae* 35 (1): 29- 40.

Khater, H.F. (2011). Biorational Insecticides for integrated pest management. *Benha Veterinary Medical Journal*, In press.

Khater, H.F., Hanafy, A.M., Abdel- Mageed, A.D., Ramadan, M.Y., & El- Madawy, R.S. (2011). The insecticidal effect of some Egyptian plant oils against *Lucilia sericata* (Diptera: Calliphoridae), *International Journal of Dermatology* 50 (2): 187–194.

Khater, H.F., Ramadan, M.Y. & El- Madawy, R.S. (2009).The lousicidal, ovicidal, and repellent efficacy of some essential oils against lice and flies infesting water buffaloes in Egypt, *Veterinary Parasitology* 164 (2-4): 257-266.

Khater, HF. & Shalaby, AA. (2008). Potential of biologically active plant oils for control mosquito larvae *Culex pipiens* (Diptera: Culicidae) from an Egyptian locality, *Revista do Instituto de Medicina Tropical de Sao Paulo* 50 (2): 107-112.

Khater, HF. (2003). *Biocontrol of some insects*. PhD thesis, Zagazig University: Benha Branch, Egypt.

Kirkland, BH., Cho, E. & Keyhani, NO. (2004). Differential susceptibility of *Amblyomma maculatum* and *Amblyomma americanum* (Acari:Ixodidea) to the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae,* Biological Control 31 (3): 414-421.

Kleeberg, H. (2006). Demands for plants protection products- Risk assessment botanicals and semiochemicals REBECA workshop, Brussels, 13–14 June; http://www.rebeca.net.de/downloads/Risk%20assessment%20botanicals%20semiochemicals%20Minutes.pdf.

Knols, BG., Bossin HC., Mukabana, WR., & Alan S. Robinson, AS. (2007). Transgenic mosquitoes and the fight against malaria: managing technology push in a turbulent GMO world, *The American Journal of Tropical Medicine and Hygiene* 77 (Suppl 6): 232–242.

Krasnoff, S.B., Watson, D.W., Gibson, D.M. & Kwan, E.C. (1995). Behavioral Effects of the Entomopathogenic Fungus, *Entomophthora muscae* on its Host *Musca domestica*: Postural Changes in Dying Hosts and Gated Pattern of Mortality, *Journal of Insect Physiology* 41 (10): 895-903.

Kumar, P., Singh, HP. & Poehling, H. (2010). Effects of neem on adults of *Eretmocerus warrae* (Hym., Aphelinidae), a parasitoid of *Bemisia tabaci* (Hom., Aleyrodidae) in tropical horticulture systems, *Journal of Plant Diseases and Protection* 117 (6): 273–277.

Kumar, U. (2002). Neem as a potential biopesticide and soil conditioner, *Agrobios Newsletter* 1(6): 8–12.

Lacey, L.A. & Kaya, H.K. (2007). *Field manual of techniques in invertebrate pathology*. 2nd Ed. Springer. Dordrecht, The Netherlands

Leal, WS., Blomquist, GJ. & Vogt, RG. (2003). Proteins that make sense. *Insect Pheromone Biochemistry and Molecular Biology*. Elsevier, Amsterdam.

Leger, R.J.S. (2008). Studies on adaptations of *Metarhizium anisopliae* to life in the soil, *Journal of Invertebrate Pathology* 98: 271-276

Leighton, TE., Marks, D. & Leighton, F. (1981). Pesticides: Insecticides and fungicides are chitin synthesis inhibitors, *Science* 213: 905-907.

Lu, FC. (1995). A review of the acceptable daily intakes of pesticides assessed by the World Health Organization, *Regulatory Toxicology and Pharmacology* 21: 351-364.

Lukšienė Z., Kurilčik N., Juršėnas S., Radžiutė, S. & Būda V. (2007). Towards environmentally and human friendly insect pest control technologies: Photosensitization of leafminer flies *Liriomyza bryoniae, Journal of Photochemistry and Photobiology B: Biology* 89: 15–21.

Lövei, GL., Andow, DA. & Arpaia, S. (2009). Transgenic Insecticidal Crops and Natural Enemies: A Detailed Review of Laboratory Studies, *Environmental Entomology* 38 (2): 293-306.

Maia, MF. & Moore, SJ. (2011). Plant-based insect repellents: a review of their efficacy, development and testing, *Malaria Journal* 10 (Suppl 1): S11.

Makonde, H.M., Lenga, F.K., Masiga, D., Mugo, S. & Boga, H.I. (2010). Effects of *Bacillus thuringiensis* CRY1A(c) d--endotoxin on growth, nodulation and productivity of beans [*Phaseolus vulgaris* (L.) and siratro (*Macroptilium atropurpureum* DC.)], *African Journal of Biotechnology* 9 (1): 017-024.

Mangan, RL. & Moreno, DS. (2001). PhotoActive Dye Insecticide Formulations: Adjuvants Increase Toxicity to Mexican Fruit Fly (Diptera: Tephritidae), *Journal of Economic Entomology* 94 (1):150-156.

Marvier, M., C. McCreedy, J. Regetz, & P. Kareiva. (2007). A meta-analysis of effects of Bt cotton and maize on non-target invertebrates, *Science* 316: 1475- 1477.

McCutchen, B.F., Hoover, K., Preisler, H.K., Betana, M.D., Herrmann, R., Robertson, J.L. & Hammock, B.D. (1997). Interaction of recombinant and wild-type baculoviruses with classical insecticides and pyrethroid-resistant tobacco budworm (Lepidoptera: Noctuidae), *Journal of Economic Entomology* 90: 1170 - 80.

McNeil J. (2010).*Viruses as biological control agents of insect pests*, organic agriculture. E:\1 chapter book 2 microbial control\McNeil 2010 Viruses as biological control agents of insect pests - eXtension.mht

Mehlhorn, H. (2011). Nature Helps, Parasitology Research Monographs 1, Springer-Verlag Berlin Heidelberg.

Merrriam, TL. & Axtell, RC. (1982). Evaluation of the entomogenous fungi *Culicinomyces clavosporus* and *Coelomomyces giganteum* for control of the salt marsh mosquito, *Aedes taeniorhynchus, Mosquito News* 42: 594-602.

Metchnikoff, E. (1879). Zur Lehre über Insektenkrankheiten, *Zoologische Anzeiger*, 3: 44-47.

Miles, C., Blethen, C. & Gaugler, A. (2000). *Using beneficial nematodes for crop insect pest control.* Washington State University Cooperative Extension. PNW544

Mochi, DA., Monteiro, AC., Machado ACR. & Yoshida, L. (2010). Efficiency of entomopathogenic fungi in the control of eggs and larvae of the horn fly *Haematobia irritans* (Diptera: Muscidae), *Ve*terinary Parasitology 167 (1): 62-66.

Moller, A.P. (1993). A fungus infecting domestic flies manipulates sexual behaviour of its host, *Behavioral Ecology and Sociobiology* 33: 403- 407.

Mordue Luntz, A.J. (2003). Arthropod semiochemicals: mosquitoes, midges and sealice, *Biochemical Society Transactions* 31 (1): 128-133.

Mordue, A.J. & Blackwell, A. (1993). Azadirachtin : an update, *Journal of Insect Physiology* 39: 903-924.

Morgan, ED. (2009). Azadirachtin, a scientific gold mine, Bioorganic & Medicinal Chemistry 17(12): 4096-4105.

Moscardi, F. (1999). Assessment of the application of baculoviruses for control of Lepidoptera, *Annual Review of Entomology* 44: 257–289.

Naumann, K. & Isman, M.B. (1996). Toxicity of neem *(Azadirachta indica* A. uss) seed extracts to larval honeybees and estimation of dangers from field applications, *American Bee Journal* 136:518–20

Nerio, LS., Olivero-Verbel, J. & Stashenko E. (2010). Repellent activity of essential oils: a review, Bioresource Technology 101(1): 372-378.

Nestrud, LB. & Anderson, RL. (1994). Aquatic safety of *Coelomomyces giganteum*: effects on freshwater fish and invertebrates, *Journal of Invertebrate Pathology* 64: 228-233.

O'Brien, D.J. and Fahey, G. (1991). Control of fly strike in sheep by means of a pour-on formulation of cyromazine, *Veterinary Record* 129: 351–353.

Orduz, S. & Axtell, RC. (1991). Compatibility of *Bacillus thuringiensis var. israelensis and Bacillus sphaericus with the fungal pathogen Coelomomyces giganteum (Oomycetes:Lagenidiales), Journal of the American Mosquito Control Association 7: 188-193.*

Owolade, OF., Ogunleti, DO. & Adenekan, MO. (2008). Titanium Dioxide affects disease development and yield of edible cowpea, *Electronic journal of Environmental Agriculture and Food chemistry* 7(50): 2942-2947.

Palevitch, D. & Craker, L.E. (1994). Volatile oils as potential insecticides. *Herb, Spice and Medicinal Plant Digest* 12: 1–5.

Patil, SA. (2009). *Economics of agri poverty: Nano-bio solutions.* I*ndi*an Agricultural Research Institute, New Delhi, Indian.

Pinto, MC. & do Prado, AP. (2001). Resistance of *Musca domestica* L. Populations to Cyromazine (Insect Growth Regulator) in Brazil, Memórias do Instituto Oswaldo Cruz 96(5): 729-732.

Pitasawat, B., Choochote, W., Tuetun, B., Tippawangkosol, P., Kanjanapothi, D., Jitpakdi, A. & Riyong, D. (2003). Repellency of aromatic turmeric *Curcuma aromatica* under laboratory and field conditions, *Journal of Vector Ecology* 28 (2): 234-240.

Pretty, J. (2009). *The Pesticide Detox, Towards a More Sustainable Agriculture*. Earthscan, London.

Price, DN. & Berry, MS. (2006). Comparison of effects of octopamine and insecticidal essential oils on activity in the nerve cord, foregut, and dorsal unpaired median neurons of cockroaches, *Journal of Insect Physiology* 52: 309-319.

Priestley, C.M., Burgess, I.F. & Williamson, E.M., (2006). Lethality of essential oils constituents towards the human louse *Pediculus humanus*, and its eggs, *Fitoterapia* 77, 303–309.

Priestley, CM., Williamson, EM., Wafford, KA. & Sattelle, DB. (2003). Thymol, a constituent of thyme essential oil, is a positive allosteric modulator of human GABAA receptors and a homo-oligomeric GABA receptor from *Drosophila melanogaster*, *Britich Journal of Pharmacology* 140:1363–7278.

Quesada-Moraga, E., Ruiz-Garcia, A. & Santiago-Alvarez, C. (2006). Laboratory evaluation of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* against

puparia and adults of *Ceratitis capitata* (Diptera : Tephritidae), *Journal of Economic Entomology* 99: 1955-1966.

Ragaei, M. & Khater, H.F. (2004). *Laser and photobiology applications: Photosensitizers as photopesticides, Phototherapy and phototoxicity in animals*. Workshop on Laser Chemistry and Applications Materials and Biomedical Research. National Research Center, Cairo, October 2-5, 2004.

Ragaei, M., Abdel- Latef, H. & Khater, H.F. (2004) Using of *Bacillus sphaericus* as microbial control agent against mosquito larvae. 1s1 international scientific Conference of toxicology 18- 19 Dec. (2004) Faculty of Pharmacy, 6th October University.

Ravensberg, WJ. (2011). *A Roadmap to the Successful Development and Commercialization of Microbial Pest Control Products for Control of Arthropods*. Springer.

Regnault-Roger C. (2011). Risks of Biocontrol Agents Containing Compounds of Botanical Origin or Semiochemicals, in Ehlers R. (ed.), Regulation of Biological Control Agents, Springer, Part 2, pp. 215-242, DOI: 10.1007/978-90-481-3664-3_10

Renn, N. (1995). Mortality of immature houseflies (*Musca domestica* L.) in artificial diet and chicken manure after exposure to encapsulated entomopathogenic nematodes (Rhabditida: Steinernematidae, Heterorhabditidae), *Biocontrol Science and Technology* 5: 349-359.

Renn, N. (1998).The efficacy of entomopathogenic nematodes for controlling housefly infestations of intensive pig units, *Medical and Veterinary Entomology* 12: 46–51.

Retnakaran, A., Hiruma, K., Palli, S.R. & Riddiford, L.M. (1995). Molecular analysis of the mode of action of RH-5992, a lepidopteran-specific, non-steroidal ecdysteroid agonist, *Insect Biochemistry and Molecular Biology* 25: 109–117.

Retnakaran, A., Macdonald, A., Tomkins, W.L., Davis, C.N., Brownright, A.J., et al., 1997. Ultrastructural effects of a non-steroidal agonist, RH-5992, on the sixth instar larvae of spruce budworm, *Choristoneura fumiferana, Journal of Insect Physiology* 43: 55–68.

Roffey J. (1968). The occurrence of the fungus *Entomophthora grylli* Fresenius on locusts and grasshoppers in Thailand, *Journal of Invertebrate Pathology* 11: 2377 241.

Salako, EA., Anjorin, ST., Garba, CD., & Omolohunnu, EB. (2008). A review of neem biopesticide utilization and challenges in Central Northern Nigeria, *African Journal of Biotechnology* 7 (25): 4758-4764.

Samuels, R. I., Coracini, D.L.A., Martins dos Santos, C.A. & Gava, CTA. (2002). Infection of *Blissus antillus* (Hemiptera: Lygaeidae) eggs by the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana, Biological Control* 23: 269–273.

Schmutterer, H. (1990). Properties and potential of natural pesticides from the neem tree, *Azadirachta indica, Annual Review of Entomology* 35: 271–297.

Schmutterer, H. (1995). In: The neem tree *Azadirachta indica* (A. Juss) and other meliaceous plants: sources of unique natural products for integrated pest management, medicine, industry and other purposes, VCH Verlagsgesellschaft mbH, Weinheim, Germany, p. 696.

Scholte, EJ., Knols, BGJ., Samson, RA. & Takken, W. (2004). Entomopathogenic fungi for mosquito control: A review, *Journal of Insect Science* 4(19): 1-24

Schuster, D. J. & Stansly, P.A. (2006). Chemical Control: Biorational Insecticides, pp199-210. *In* J. L. Gillett, H. N. HansPetersen, N. C. Leppla and D. D. Thomas (Eds.) Grower's

IPM Guide for Florida Tomato and Pepper Production. University of Florida, Extension. 206 p.

Shalaby, A.A. & Khater, H.F. (2005). Toxicity of certain solvent extracts of *Rosmarinus officinalis* against *Culex pipiens* larvae, J*ournal of the Egyptian- German Society of Zoology* (48E): 69- 80.

Sharma, RN. (1993). The utilization of essential oils and some common allelochemic constituent for non-insecticidal pest management strategies. In: *Newer Trends Essent-51*. New Delhi (India): Tata McGraw Hill.

Shathele, M.S. (2009). New isolate media of the mosquito pathogenic fungus *Lagenidium giganteum* (Oomycetes: Lagenidiales) for fungal maintenance and zoospores release. Journal of Entomology 6 (3):167-175.

Singh, A., Parmar, N. & Kuhad, RC. (2011). *Bioaugmentation, Biostimulation and Biocontrol*, Springer-Verlag Berlin Heidelberg.

Smart Jr., GC. (1995). Entomopathogenic Nematodes for the Biological Control of Insects, Supplement to the *Journal of Nematology* 27(4S): 529-534. 1995.

Staal, G.B. (1986). Anti-juvenile horone agents, *Annual Review of Entomology* 31:391-429.

Steenberg, T., Jespersen, J.B., Jensen, K.M.V., Nielsen, B.O. & Humber, R.A. (2001). Entomopathogenic Fungi in Flies Associated with Pastured Cattle in Denmark, *Journal of Invertebrate Pathology* 77: 186–197.

Stephen, K. & Kurtböke, DI. (2011). Screening of Oomycete Fungi for Their Potential Role in Reducing the Biting Midge (Diptera: Ceratopogonidae) Larval Populations in Hervey Bay, Queensland, Australia. *International Journal of Environmental Research and Public Health* 8: 1560-1574.

Sun, X., Wang, H., Sun, X., Chen, X., Peng, C., Pan, D., et al. (2004). Biological activity and field efficacy of a genetically modified *Helicoverpa armigera* SNPV expressing an insect-selective toxin from a chimeric promoter, *Biological Control* 29:124-137.

Sur B, Bihari V, Sharma A, Joshi AK. (2001). Studies on physiology, zoospore morphology and entomopathogenic potential of the aquatic oomycete: *Coelomomyces giganteum*. *Mycopathologia* 154: 51-54.

Szewczyk, B, Rabalski, L., Krol E., Sihler W. & Lobo de Souza, M. (2009). Baculovirus biopesticides - a safe alternative to chemical protection of plants, *Journal of Biopesticides* 2 (2): 209-216.

Szewczyk, B., Lobo de Souza, M., Batista de Castro, ME., Moscardi, ML. & Moscardi, F. (2011). *Baculovirus* Biopesticides. *In M. Stoytcheva (ed.), Pesticides - Formulations, Effects, Fate*, InTech. p. 25-36.

Taylor, MZ. (2001). Recent Developments in Ectoparasiticides, *The Veterinary Journal* 161(3):253-268.

Toloza, A., Lucía, A., Zerba, E., Masuh, H. & Picollo, MI. (2008) Interspecific hybridization of Eucalyptus as a potential tool to improve the bioactivity of essential oils against permethrin-resistant head lice from Argentina, *Bioresour Technology* 99:7341–7347.

Tomlin, C.D.S. (2000). *The Pesticide Manual*, 12th edn. British Crop Protection Council Publications.

Torney F (2009). *Nanoparticle mediated plant transformation*. Emerging technologies in plant science research. Interdepartmental Plant Physiology Major Fall Seminar Series. Phys. p. 696.

Tripathi, AK., Upadhyay, S., Bhuiyan, M. & Bhattacharya, P.R. (2009). A review on prospects of essential oils as biopesticide in insect-pest management, *Journal of Pharmacognosy and Phytotherapy* 1(5): 052-063.

Tunaz, H. (2004). Insect Growth Regulators for Insect Pest Control, *Turkish Journal of Agriculture and Forestry* 28: 377-387.

UNEP. (2000). *The Montreal Protocol on Substances that Deplete the Ozone layer*. United Nations Environment Programme, Nairobi, Kenya. P. 1-54 (www.unep.org/ozone).

van Emden, H. F. & Service, M.W. (2011). Pest and Vector Control. Cambridge University Press, 2001. Cambridge Books Online. Cambridge University Press. 31 http://dx.doi.org/10.1017/CBO9780511616334

Vasconcelos S.D.V., Cory J.S., Wilson K.R., Sait S.M. & Hails R.S. (1996). The effect of baculovirus infection on the mobility of *Mamestra brassicae* L. (Lepidoptera: Noctuidae) larvae at different developmentalstages, Biological Control 7: 299–306.

Veal, L. (1996). The potential effectiveness of essential oils as a treatment for headlice, *Complement ther nurs midwifery* 2: 97–101.

Vidhyalakshmi, R., Bhakyaraj, R. & Subhasree, RS. (2009). Encapsulation the Future of Probiotics-A Review, *Advances in Biological Research* 3(3-4): 96-103.

Vreysen, M.J.B., Robinson, A.S. & Hendrichs, J. (2007). *Area-wide control of insect pests, from research to field implementation*. Springer, Dordrecht, The Netherlands.

Walker, KR. & Welter, SC. (2010). Potential for outbreaks of leafrollers (Lepidoptera: Tortricidae) in California apple orchards using mating disruption for codling moth suppression, *Journal of Economic Entomology* 94:80.

Wan, MT., Watts, RG., Isman, MB. & Strub, R. (1996). An evaluation of the acute toxicity to juvenile Pacific northwest salmon of azadirachtin, neemextract and neem-based products, *Bulletin of Environmental Contamination and Toxicology* 56:432–439.

Ware, GW. & Whitacre, DM. (2004). *The Pesticide Book*, 6th Ed. Meister Media Worldwide, Willoughby, Ohio.

Watson, G.S., Watson, JA., Hu, S., Brown, CL., Cribb, BW. & Myhra, S. (2010). Micro and nanostructures found on insect wings - designs for minimising adhesion and friction, *International Journal of Nanomanufacturing* 5:112-128.

Welter, SC., Millar, CG., Cave, F., Van Steenwyk, RA. & Dunley, J. (2005). Pheromone mating disruption offers selective management options for key pests, *California Agriculture 59*(1):16-22.

Wirth, MC., Park, HW., Walton, WE., & Federici, BA. (2005). Cyt1A of *Bacillus thuringiensis* delays evolution of resistance to Cry11A in the mosquito *Culex quinquefasciatus*, *Applied and Environmental Microbiology* 71:185–189

Witzgall, P. (2001). Pheromones - future techniques for insect control? *Pheromones for Insect Control in Orchards and Vineyard, IOBC wprs Bulletin* 24 (2): 114-122.

Yang, FL., Li, XG., Zhu, F. & Lei, CL. (2009). Structural Characterization of Nanoparticles Loaded with Garlic Essential Oil and Their Insecticidal Activity against *Tribolium*

castaneum (Herbst) (Coleoptera: Tenebrionidae), *Journal of Agricultural and Food Chemistry* 57(21): 10156-10162.

Zabalgogeazcoa. I., Oleaga A. & Pérez-Sánchez, R. (2008). Pathogenicity of endophytic entomopathogenic fungi to *Ornithodoros erraticus* and *Ornithodoros moubata* (Acari: Argasidae), Veterinary Parasitology 158 (4): 336-343.

Zhou, M.Z., Sun, H.C., Hu, Z.H.& Sun, X.L. (2004). SOD enhances infectivity of *Helicoverpa armigera* single nucleocapsid nucleopolyhedrosis against *H.armigera* larvae, *Virologica Sinica*, 18: 506-507.

3

Ecological Impacts of Insecticides

Francisco Sánchez-Bayo
Centre for Ecotoxicology, University of Technology Sydney, Australia

1. Introduction

Substances capable of killing insects and other pests were discovered by the Persians some 2500 years ago, but it was the Green Revolution since the 1950s that fostered the development of new synthetic pesticides to cope with the demands of the explosive human population. We have now reached 7 billion people and managed to reduce starvation in many underdeveloped countries. In fact, the world's agricultural productivity has increased 2.6-fold but the arable area has increased only by 10%, mostly at the expense of our forests (FAO 2004).

Synthetic insecticides applied to a full range of crops soon started to have side effects in the surrounding natural ecosystems. The direct effects of insecticides on arthropod communities, and the birdlife that depends on them, was brought into question by Rachel Carson as early as 1962. It is not coincidence that the birth of the environmental movement and ecotoxicology was thus linked from its very beginnings to the widespread use of synthetic pesticides in agriculture and forestry. These fears became reality when the unrestrained over-use of these 'wonder chemicals' in Indonesia and the Philippines led to the collapse of their rice crops in the early 1970s. The destruction of predators within the complex food web of rice ecosystems released the pressure on pest species such as plant hoppers (*Nilaparvata lugens*), which devastated the crops. This love affair with chemicals ended when the Indonesian government banned many insecticides and restricted the use of other pesticides (Daryanto 1998). From then onwards farmers were encouraged to foster natural enemies that can control or at least avoid the onset of pests in agricultural ecosystems. Pesticides were not to be used alone, but in combination with other biological and agronomic practices that protect the ecosystem. Thus, the Integrated Pest Management (IPM) strategies, first proposed by the Food and Agricultural Organisation (FAO) of the United Nations in October 1965, started to replace the conventional Green Revolution practices. However, to grow enough food for us all is a major challenge faced by modern agriculture. Because the relentless expansion of our cities often demands that old, fertile agricultural land be converted to urban dwellings, our planet is actually running out of suitable land to grow food for the future generations.

This chapter deals with the known ecological impacts of insecticides on terrestrial and aquatic ecosystems, without consideration of the economic benefits that they may have. We must accept that pest control and disease eradication are complex issues that will never be resolved without causing problems to the environment –and there are not simple solutions to complex problems. Our aim should be to minimise the environmental impacts to levels that are acceptable by the society and bearable by the ecosystems.

2. Use of insecticides and acaricides

Worldwide use of insecticides is estimated at over one million tons annually (FAO 2010), comprising some 28% of the total amount of pesticides used. Most of the insecticides are still produced and used in North America, Europe and Japan, but countries such as China and India are expected to take the lead in the coming years. In fact, while insecticide usage has remained stable or even declined in developed countries due to regulatory restrictions in favour of new agricultural trends (e.g. GM crops, organic agriculture), developing countries are producing large quantities of old and highly toxic insecticides that have already run out of patent. For example, insecticides make up 7% of the total pesticides used in Europe, 19% in the USA, but 38% in Asian countries and 86% in Africa.

Currently, there are about 460 chemical compounds used for controlling insects, mites and other invertebrates worldwide. Most of them are used as insecticides (60%) or pheromones to attract insects (14%), while some 24% are used to control mites (acaricides), and only a few to eliminate nematodes (nematicides) and snails (molluscicides). Here, all these products are referred to as insecticides so long as they are used for: i) protection of crops and produce from pests; ii) prevention of diseases in livestock and human populations; and iii) control of nuisance insects in urban settings and industrial facilities.

Farmers are by far the main consumers of insecticides. Indeed, about one fifth of the world land is dedicated to agriculture, with 12% of the area being cultivated and the remaining area used for livestock. In some European countries and in the USA, that proportion could be as high as 25% of the land. Most of the insecticides are applied directly to the crops to protect them against insect pests, but a small proportion is used to protect the harvested produce (mostly grain) in storage facilities before their distribution and transport to the market. The overall economic costs of insecticides in developed countries, including the actual products plus the cost of monitoring to ensure food safety, are between 1-2% (UK) and 4% (USA) of the farm revenues (Pimentel 2005). However, in crops such as cotton insecticides can represent up to 20% of the running costs of a farm. Human health costs and fatalities should also be added to these economic costs.

Insecticides are also essential tools to eradicate disease vectors in tropical and subtropical regions. Tse-tse fly (*Glossina* sp.) and small blackfly (*Simulium damnosum*) eradication programs, conducted since the 1950s with insecticides, have reduced the incidence and risk of nagana and sleeping sickness (trypanosomiasis), and river blindness disease (onchocerciasis) among African peoples. Mosquito control using insecticide impregnated nets is very effective in preventing the spread of malaria and West Nile virus in tropical and subtropical regions. As a result of using new chemicals and improved eradication techniques the impacts on non-target organisms have been ameliorated significantly in recent years. This is a case where the risks to human health take priority over environmental impacts, which are recoverable and in the long-term have proven to be small compared to game extermination or bush clearing. In a similar way, prevention of diseases in livestock is achieved by pour-on and dip techniques using insecticides.

3. Toxicity of insecticides at the individual level

Organisms are affected by the toxicity of insecticides firstly upon direct exposure to these chemicals. The route of exposure is essential in determining the effects at the individual level. For example, after spraying a field crop with an organophosphorus (OP) insecticide,

birds that receive the spray directly on the feathers may get more exposure and die sooner than those that simply inhale its vapour, or those that eat contaminated grain or insects. This is because the total dose of OP insecticides acquired through the skin or feathers is higher than that inhaled or ingested, and 'it is the dose that makes the poison' (Paracelsus). Ingestion, on the other hand, may be a more important route of exposure in the case of organochlorine (OC) insecticides because these are persistent and accumulate in the body – so the exposure route depends to a large extent on the chemistry of each compound.

Category	Crustaceans	Bees	Worms	Fish	Mammals, birds*
	µg/L	µg/bee	mg/kg soil	mg/L	mg/kg b.w.
Highly toxic (XXX)	<10	<1	<10	<1	<50
Moderately toxic (XX)	10-100	1-10	10-100	1-10	50-500
Slightly toxic (X)	100-1000	10-100	100-1000	10-100	500-5000
Non-toxic (0)	>1000	>100	>1000	>100	>5000

* Acute oral LD50

Table 1. Toxicity categories for different taxa based on LC50 or LD50 values.

The toxicity and specificity of insecticides is a consequence of their biochemical mode of action at the cellular or physiological level in the organisms. Table 1 shows the toxicity categories for several commonly assessed taxa of non-target organisms. While the toxicity is determined by the internal dose required to cause the death of an organism, the specificity depends on the physiological mechanisms targeted by the insecticide, which can either vary substantially among taxa (selective) or be the same for all animals (broad-spectrum). Although originally intended to control insect pests, broad-spectrum insecticides are hazardous poisons to all kinds of animals, even if their lethal doses are necessarily higher for larger animals than for small insects. The mode of action of insecticides is well known for most compounds (see Chapter XX), and can be summarised in the following five major groups:

3.1 Neurotoxic insecticides

Their target is one of the five neurotransmitter systems –particularly cholinergic– found in the neuronal system of arthropods, which are either activated (agonist action) or inhibited (antagonist action) by the insecticide. Break down of the neuronal activity causes brain death or affects the motor system through paralysis, convulsions, hyperactivity and spasms.

a. Acetylcholinesterase (AChE) inhibitors comprise the organophosphorus (e.g. chlorpyrifos, dimethoate and 60 others) and carbamate (e.g. aldicarb, methomyl, pirimicarb and 25 others) insecticides. Since AChE is the main enzyme of nicotine and muscarine receptors of neurons and muscular junctions in animals, these are broad-spectrum and very toxic poisons, especially to mammals and birds.
b. γ-aminobutyric acid receptors (GABA-R) are located in the post-synaptic dendrites of the central nervous system in all animals, but in arthropods also in the neuromuscular junctions and ganglia. The avermectins (e.g. abamectin) are particularly toxic to arthropods (i.e. zooplankton, macro-crustaceans and spiders) but not so much to vertebrates (Table 2); the cyclodiene OC insecticides (e.g. endosulfan) are more toxic to terrestrial animals and fish, and fipronil somewhere in between.
c. Nicotine acetylcholinesterase receptors (nACh-R) are located in the post-synaptic dendrites of all neurons in the brain, spinal cord, ganglia, and muscular junctions.

Nicotine, neonicotinoids (e.g. imidacloprid, thiacloprid) and spinosad activate it, causing hyperactivity and death. The dithiols (e.g. cartap) block it, causing paralysis and eventually death. Since the nACh-R is different in vertebrates, these insecticides are quite specific to arthropods, but dithiols are very toxic to birds as well.

d. Sodium channels are located in all neuronal membranes. In arthropods, fish and other aquatic animals these channels are kept open by DDT and analogue organochlorines, pyrethrins and synthetic pyrethroids (e.g. deltamethrin and 45 others), thus causing loss of nervous impulse (knockdown) and eventually death, whereas indoxacarb blocks sodium channels. Pyrethroids are the most toxic insecticides to fish and crustaceans (Table 2).
e. Octopamine receptor: amitraz inhibits this receptor involved in energy demanding activities in invertebrates (e.g. jumping, flying, light emission, etc), which is modulated by the dopaminergic system. In vertebrates this receptor is associated with noradrenalinergic systems, so with the exception of fish amitraz is not very toxic to vertebrates.

3.2 Respiration inhibitors

All of them are broad-spectrum insecticides that disrupt the mitochondrial oxidative phosphorylation system common to most animals. Some uncouple the complex I (e.g. rotenone), whereas dicofol targets complex II and others complex III, but for most (e.g. chlorfenapyr, diafenthiuron, DNOC, etc.) the exact target is unknown. In any case, they are very toxic to fish and worms, but not so toxic to vertebrates. ATPase inhibitors such as the organotins and propargite, which are used as acaricides, are quite toxic to fish and crustaceans. Fumigants are particularly toxic, especially to worms and birds, and so is chlorfenapyr (Table 2).

3.3 Growth inhibitors and regulators (IGR)

These are very selective insecticides that break the life-cycle of arthropod development and metamorphosis, so they are quite harmless to vertebrates and worms (Table 2).

a. Chitin biosynthesis inhibitors: benzoylureas (e.g. diflubenzuron), cyromazine and buprofezin disrupt growth in all arthropods, since they all have an exoskeleton made of chitin. Crustaceans, however, can be seriously affected by the residues in waters.
b. Mite growth disruptors (e.g. clofentezine) are selective to this taxon, and practically non-toxic for any other taxa except for crustaceans.
c. Ecdysone agonists: the steroidal hormone ecdysone, which prompts moulting in arthropods, is mimicked by azadirachtin and diacylhydrazines (e.g. tebufenocide). These compounds cause premature moulting in the larval stages of some insect taxa such as Lepidoptera –but not bees–, thus preventing them from reaching the adult stage in due time. Very selective and non-hazardous to other taxa.
d. Juvenile hormone analogues (JH): methoprene, hydroprene, kinoprene, pyriproxyfen and fenoxycarb are agonists that keep all insects (including bees) in their larvae stage, thus preventing the pupa to moult into adult. Selective, but can affect zooplankton too.

3.4 Stomach poisons

Cryolite, sulcofuron sodium and the toxins produced by *Bacillus thuringiensis* (Bt) destroy the midgut tissues in caterpillars, being therefore very specific to Lepidoptera insects but quite harmless to all other animals.

Group	Mode of action	Insecticide class	Aquatic organisms			Terrestrial organisms			
			Zooplankton cladocerans	Macro-crustaceans	Fish	Bees	Worms	Birds	Mammals
Neurotoxic	AChE (-)	Carbamates	XX	XX	XX	XXX	XX	XXX	XXX
		Organophosphorus	XXX	XX	XX	XXX	XX	XXX	XXX
	GABA-R (+) (-)	Avermectins	XXX	XX	0	XXX	X	X	XX
		Cyclodiene OC, fipronil	XX	XXX	XXXa	XXX	XXX	XX	XX
	nACh-R (+) (-)	Neonicotinoids, spinosad	0	0a	0	XXX	XX	X	X
		Dithiols	0	?	XX	X	?	XXX	XX
	Na channel (+) (-)	Pyrethroids, DDT	XXX	XXX	XXX	XXX	X	0	X
		Indoxacarb	X	?	XXX	XX	0	XX	X
	Octopamine-R (-)	Amitraz	XX	?	XXX	X	0	X	X
Respiration inhibitors	ATPase (-)	Organo-metallic, propargite	XX	XX	XXX	0	X	0	X
	e^- transport (-)	Miscellaneous	XX	X	XXX	XX	XX	X	X
	Other	Fumigants	X	XX	XX	X	XXX	XXX	XX
Growth inhibitors	Chitin (-)	Benzoylureas, buprofezin, others	XXX	XXX	X	X	X	X	0
	Mite growth (-)	Miscellaneous	XX	?	X	0	0	0	0
	Hormone mimics (+)	Ecdysone agonists	0	0	X	0	0	0	0
		Juvenile hormones	XX	?	XX	XX	X	0	0
Stomach poisons	Membrane disruption	*B. thuringiensis* (Bt), sulcofuron, cryolite	0	?	X	?	?	0	0
Others	Detoxification (-)	Synergists	0	?	X	X	XX	0	0
	Lures	Pheromones	0	0	X	?	?	0	0
	Unknown	Miscellaneous	X	0	XXX	X	X	X	0

* Based on geometric mean LC50s or LD50s for surrogate species
a Fipronil is non-toxic to fish, and imidacloprid moderately toxic to all crustaceans except cladocerans (waterfleas)

Table 2. Comparative toxicity* of insecticides to non-target organisms, according to their mode of action: agonists (+), antagonists (-). Symbols (X, 0) indicate the toxicity categories as in Table 1: question mark denotes no data available.

3.5 Other modes of action

Pymetrozine and azadirachtin have anti-feedant properties, but the mechanism involved in this action is not well understood. Synergists enhance the toxicity of other insecticides by inhibiting the cellular detoxification mechanisms (e.g. monoxygenases, cytochrome P450, etc), and are quite toxic to worms. Natural or artificial pheromones attract individuals of the same pest species (i.e. sex pheromones for females), being the most specific substances used in insect control and one of the safest for non-target organisms (Table 2).

When exposure to an insecticide is below its mortality levels, the individuals affected may undergo sublethal effects, i.e. negative side effects that are unrelated to the specific mode of action of that insecticide. Some examples are the reproduction impairment as a consequence of sperm deformity in earthworms caused by imidacloprid (Luo et al. 1999), the depressed immunological response of frogs to trematode infections (Rohr et al. 2008) and the disruption of endocrine regulatory systems in many organisms by a number of pesticides (Manning 2005). Sublethal effects are unpredictable so long as they are due to unknown physiological mechanisms. The best known sublethal mechanism is the thinning of eggshells in birds caused by accumulation of DDE and OC residues (Cooke 1973), which results in high frequency of eggs breaking and, therefore, in reproduction failure. Behavioural changes are also sublethal effects even if they may result from the neurotoxic activity of the insecticide. For example, bees exposed to low doses of permethrin are not actively involved with foraging, but spend their time rubbing legs, trembling, dancing and in self-cleaning activities (Cox and Wilson 1984). Frogs exposed to malathion have reduced predatory skills (Relyea 2004), and starlings exposed to OP insecticides neglect looking after their nestlings, thus causing early death of chicks and reproduction failure (Grue et al. 1982).

4. Impacts on non-target populations

Wild animals live in populations of few or many individuals in accordance with the distribution and behaviour of each species. Direct exposure to insecticides occurs in and around the sprayed crops, affecting usually a limited number of individuals of each population, not all. However, repeated exposures over time may have dire consequences for a given population when its reproductive capacity is impaired. Thus, population declines may result from direct toxicity, either through primary or secondary poisoning, or from sublethal effects manifested as reductions in life span, development rates, fertility, fecundity, sex ratio, and behaviour (e.g., feeding, foraging and reproduction).

4.1 Terrestrial populations

Impact of OP and carbamate insecticides on earthworms populations in agricultural fields and grasslands have been reported since the 1960s (see review in Brown 1978). Whilst all fumigants wipe out earthworms as they penetrate the deep layers of the soil, the majority of OC, OP and carbamate insecticides do not cause significant reduction of earthworm populations at normal application rates (e.g. aldrin 2.5 kg/ha). However, endosulfan, parathion-methyl and carbaryl can cause 15-60% reductions, and chlordane, heptachlor, phorate and carbofuran are so toxic to worms that they eliminate them completely, although their numbers recover within a year if they are re-colonised from the surrounding area. Recovery times are usually 60 to 80 days, but following carbofuran treatments, can take 90-105 days, and following application of the OC insecticide butachlor can be longer than a season. Replacement of those broad-spectrum insecticides with pyrethroids such as λ-

cyhalothrin has practically no impact on tropical earthworm populations because the latter class of insecticides is less toxic to earthworms (Table 2). Neonicotinoids are also safer to these organisms, but sublethal effects on earthworm burrowing activity have been observed after application of imidacloprid at 0.5-1.0 mg/kg in soil (Capowiez et al. 2006), as well as significant weight losses and 44-97% less cast production within the typical range 0.33-0.6 mg/kg of this insecticide in soil (Dittbrenner et al. 2010). Weight loss and cocoon production among earthworms are also seriously affected when concentrations of imidacloprid in the litter of treated forests exceed 3 mg/kg (Kreutzweiser et al. 2008). The latter effects obviously affect the viability of earthworm populations in the short-term.

The direct impact of insecticides on honey bees (*Apis mellifera*) was recognized as a problem since the calcium arsenate dust sprays killed entire hive colonies in the past (Brown 1978). Bees are insects, and so pyrethroid and OP insecticides are very toxic and hazardous to them (Table 2). An average of 50 poisoning incidents per year were confirmed in Great Britain between 1981-91, involving some 30 insecticides, of which triazophos, dimethoate, fenitrothion and lindane were the most common (Greig-Smith et al. 1994). Spray drift and volatilization are responsible for most of these incidents. Pyrethroids pose a high risk not just to honey bees but also to wild bumblebees (*Bombus* sp.), all of which play an important role in the pollination of many flowering plants and vegetable crops (Thompson 2001). Bees can also be killed by ingestion of contaminated nectar and pollen. The introduction in recent years of systemic insecticides such as imidacloprid has been blamed as the main cause of the declining bee colonies in France and other European countries, although not without controversy (Maxim and Sluijs 2007). Even if typical residues of imidacloprid in pollen (2.1 μg/kg for a range of plants (Bonmatin et al. 2005)) from agricultural areas treated with this insecticide are below the oral LD50 for honeybees (18 ng/bee), chronic feeding on such pollen may lead to mortality of the bees. In addition to direct mortality, imidacloprid impairs the memory and activity of worker bees, which likely result in the failure to rear their larvae in the hive. Insecticides also affect the performance of the bee colonies, with impacts ranging from odour discrimination to the loss of foraging bees due to disruption of their homing behaviour.

Spraying crops and forests with OP and carbamate insecticides can cause temporary reductions in songbird populations by directly killing some individuals. However, casualties among birds are hard to observe and quantify due to their mobility and because dead birds are difficult to find in the surrounding treated areas. Evidence of impacts comes from sublethal effects observed in debilitated birds after the spraying: brain AChE bioassays confirm the depression caused by fenitrothion and aminocarb spray formulations but overall impacts of fenitrothion spraying on forest bird populations appear to be minimal. The effects of fenthion on the breeding success of red-winged blackbirds (*Anglaius phoenicius*) in USA were negligible (Powell 1984), and spraying of malathion, carbaryl and fipronil for locust control reduced some songbird populations in Wyoming but increased others; residual effects can be more important than the acute toxicity of the compounds when considering their overall impacts on bird populations. Impacts of repellent methiocarb sprays to protect cherry orchards against birds are transient and usually confined to sublethal effects (Hardy et al. 1993). Insecticide sprays to control vector diseases can also cause declines in some populations of birds, but most effects are sublethal: i.e. when malathion was sprayed in Haiti to control malaria.

Lack of apparent direct effects on bird populations can be masked, however, by immigration of individuals from nearby areas, a compensatory effect that is difficult to gauge for most

species in the field. Small field enclosures (mesocosms) can be used to avoid this problem when experimenting with populations of small mammals, but not with birds. Thus, the impacts of endrin (0.56 kg/ha) on populations of meadow voles (*Microtus pennsylvanicus*) and deer mice (*Peromyscus maniculatus*) were studied: while both species were reduced in numbers immediately after application of the insecticide, the voles recovered and achieved higher densities in subsequent years, whereas the mice never recovered fully (Morris 1970). Similar impacts have been observed with AChE inhibitors. For example, carbaryl sprayed on oats (2.3 kg/ha) had different impacts on populations of small herbivorous mammals: while house mice (*Mus musculus*) increased in the treated fields, the population of meadow voles decreased due to a 5-6 week delay in pregnancies among their females (Barrett 1988). Sprays of azinphos-methyl (2.44 kg/ha) on pasture mesocosms reduced populations of gray-tailed voles (*Microtus canicaudus*) when the applied pasture was dry, but not when it was wet (Wang et al. 2001), perhaps because under wet conditions this OP insecticide dissipates faster, so exposure to small herbivorous mammals is minimised. Another factor that reduced the exposure of voles to this insecticide was the density and height of vegetation, with populations decreasing in shorter alfalfa pastures but not in tall prairie grasses. It should be pointed that not all OP insecticides have impacts on vole populations (i.e. acephate), even if exposure of small mammals occurs and can be demonstrated using brain or plasma bioassays.

4.1.1 Primary poisoning

Primary poisoning by consuming insecticide granules is one of the most common causes of bird mortality, since birds mistake the granules as grit for their gizzards (Mineau 2003). Phorate, terbufos, fonofos, aldicarb and carbofuran are usually applied to crops in granular form to eliminate soil larvae. Given their extreme toxicity to birds and mammals (Table 2) they are very hazardous to wildlife around corn fields and other crops. Despite efforts to reduce their risk by modifying the colour of granules, incidents on wildlife populations are probably more common than we think because most of the fatalities pass unnoticed. For example, entire populations of waterfowl were decimated when ducks and geese ate fonofos granules that were still buried in potato crops a few months after they were applied. These birds use the fallow fields as wintering grounds, and sift the buried granules in their search for food (Elliott et al. 2008). Similar incidents have occurred with other granular OP and carbamate insecticides on many occasions. Songbirds and small mammals are equally killed by granular insecticides, and granivorous birds in particular are affected by insecticide-treated seeds. In the past, seed-dressing formulations of aldrin, dieldrin, heptachlor and lindane were common and caused a large number of deaths among geese. Their replacement with OP and carbamate insecticides still produces a substantial toll on birds in Europe and other countries. For example, 15 endangered sarus cranes (*Grus antigon*) and 3 common cranes were found dead near a National Park in India in November 2000. Analysis of their stomachs revealed residues of monocrotophos at levels 0.2-0.74 mg/kg, while wheat seeds from the fields nearby had residues of 0.8-1.8 mg/kg of the same insecticide; the cranes had been eating contaminated wheat seeds and died because of the high toxicity of this OP insecticide to birds (Pain et al. 2004). Poisoning of songbirds, waders, seabirds and raptors by chlordane, lindane and DDT compounds was all too common in the past and is still occurring in North America, Japan, Russia and northern Asia, even though some OC compounds have been banned or restricted for use in agriculture (e.g. dieldrin, DDT). This is due to bioaccumulation of the persistent OC insecticides in organisms, which occurs

whenever the rate of intake is higher than the metabolic rate (degradation) of the compound in the body.

4.1.2 Secondary poisoning

Secondary poisoning of predators consuming contaminated prey often results in mortality, since the toxic dose ingested by the predator is usually higher than the residue concentrations found in soil or water: as a predator consumes contaminated prey, it accumulates more and more residues in its own body. This can continue up through the food chain through a process called biomagnification, thereby causing sickness and mortality among higher-level predators. This phenomenon is more prevalent among persistent insecticides. For example, more than 96% of lacewings (*Micromus tasmaniae*) were killed after feeding on lettuce aphid (*Nasonovia ribisnigri*) contaminated with imidacloprid applied at recommended rates (20-30 ml/1000 lettuce plants), whereas pirimicarb and pymetrazine only caused a 30-40% or less than 20% mortality, respectively, when applied also at their recommended rates (Walker et al. 2007). Most cases of secondary poisoning are reported for predatory vertebrates. Thus, OP and carbamate insecticides have been implicated in the mortality of many raptors in Europe and North America over the years (see review in Mineau et al. 1999). In countries with less or no regulation the impacts of these highly toxic insecticides can be devastating: for example, about 6000 Swainson's hawks (*Buteo swainsoni)* were found dead in the Argentinian pampas in 1995-96 after eating grasshoppers that were contaminated with monocrotophos, which was used to control a locust outbreak (Goldstein et al. 1999), and hundreds of raptors (birds of prey and owls) suffered the same fate in Israel in 1975-76 when a plague of levant voles (*Microtus guentheri*) in alfalfa fields was suppressed with a monocrotophos spray (Mendelssohn and Paz 1977). Although these are isolated incidents, it is obvious that this particular OP insecticide is too hazardous to be used in such circumstances. Populations of insectivorous vertebrates are particularly affected by secondary poisoning. For example, it is believed that populations of free-tailed bats (*Tadarida brasiliensis*) in New Mexico suffered a severe decline since 1936 due to ingestion of insects with high levels of insecticides (Clark 2001). Deer mouse (*Peromyscus maniculatus*) densities in grassland enclosures receiving 3.61 kg/ha of azinphos-methyl decreased 47% within 5 days after spraying because the mice fed on contaminated arthropods (Schauber et al. 1997). Since most reptiles and frogs are insectivorous, their exposure to pesticides is mainly through preying on contaminated insects and, in the case of snakes, other vertebrates as well. Early reports showed that sprays of 5 kg/ha of DDT to control cattle ticks in Texas resulted in the deaths of snakes, spiny lizards and probably many other reptiles in the prairie (George and Stickel 1949). However, not much information is available on the impacts that OP insecticides have on reptile populations, even if data on residues of OC insecticides and AChE inhibition can provide some indication about their levels of exposure.

4.1.3 Sublethal effects

In addition to direct mortality by poisoning with insecticides, wild populations can be reduced through sublethal effects on the reproduction of some species. For example, experimental prairie grasslands treated with diazinon reduced the reproductive capacity of the omnivorous cotton rat (*Sigmodon hispidus*) by 33-100% as it fed on contaminated arthropods, but did not affect herbivorous voles (Sheffield and Lochmiller 2001). Sublethal effects are normally detected after many years of observations that show declining trends in

the population of some species. The best known cases refer to fish-eating birds and raptors contaminated with DDE (a metabolite of DDT) and other OC insecticides. Sublethal effects of DDE were first identified by Ratcliffe (1967) in populations of peregrine falcon (*Falco peregrinus*) in Britain, which experienced an unusually high rate of hatching failure (28%) due to breakage of their eggshells. Although OC insecticides had already been linked to declining populations of golden eagles (*Aquila chrysaetos*) in Scotland, the mechanism by which DDT and cyclodiene insecticides were affecting birds of prey was not solved until it was found that DDE metabolite interfered with calcium deposition during the eggshell formation, leading to thinner shells that were prone to breakage. The high breeding failure among Spanish imperial eagles (*Aquila adalberti*) between 1972 and 2003 was mainly due to DDT residue accumulation in their eggs (Hernández et al. 2008). This insecticide is also a contributing factor for the declining populations of booted-eagles (*Hieraetus pennatus*), goshawk (*Accipiter gentilis*), and possibly other birds of prey in Spain, despite the many years elapsed since DDT was banned from use in agriculture. Cyclodiene insecticides were also involved in reproductive failures. For example, accumulation of dieldrin in British sparrowhawks (*Accipiter nisus*) during the 1960-70s resulted in population declines of up to 60% per year due to a combination of direct mortality and hatching failure; after the cyclodiene insecticides were banned, the sparrowhawk populations recovered (Sibly et al. 2000). Among fish-eating birds, the hatching failure (32% eggs) of double-crested cormorants (*Phalacrocorax auritus*) in Lake Michigan during the 1994-95 seasons was mainly due to DDE residues, not to dieldrin or polychlorinated biphenyls (PCB), even if DDT was banned for use in the area some 25 years earlier (Custer et al. 1999). Similar conclusions have been drawn from studies on populations of brown pelicans (*Pelecanus occidentalis*) in South Carolina, Louisiana and Florida during the 1960-70s, of common terns (*Sterna hirundo*) in the Canadian Great Lakes during the 1980s, and great blue herons (*Ardea herodias*) in the upper Mississippi river during the 1990s. However, the effect of DDE residues in the reproductive success of black-crowned nightheron (*Nycticorax nycticorax*) in New England and North Carolina colonies appeared to be minimal (Custer et al. 1983). Many other predatory bird populations have been affected by DDT, but fortunately most of them are now recovering after this and other OC insecticides were banned in most developed countries.

Endocrine disruption by insecticides was found when populations of American alligator (*Alligator mississippiensis*) from Lakes Apopka, Griffin, and Okeechobee in central and south Florida were investigated in 1984 after a spill of dicofol and DDT. Although OC residues in their eggs appeared to bear not relation to the hatching success of the colonies, further research linked the declining population trends in lake Apopka to developmental abnormalities in the gonads, which were due to sex hormonal imbalances that resulted in fewer males being born (Guillette et al. 1995), and males having poorly organized testes and abnormally small phalli. The combination of sublethal levels of DDT, its metabolite DDE and PCB residues in bald eagles (*Haliaetus leucocephalus*) from the Great Lakes region of North America has resulted in the so-called Great Lakes embryo mortality, edema and deformities syndrome (GLEMEDS), of which the most obvious consequence is a reproduction impairment that hampers the viability of populations of this endangered species. Various endocrine disruptive effects have been found among a wide range of species and taxa exposed to some insecticides (see review in Manning 2005). For example, hyperthyroidism in birds with sublethal levels of p,p'-DDT was shown as early as 1969, and 16 day-old chicks of tree swallow (*Tachycineta bicolor*) showed abnormal thyroid development after being exposed to carbamate, OP and pyrethroid insecticides sprayed on

apple orchards (Bishop et al. 1998). However, since these effects are subtle and difficult to assess, their impacts on wildlife populations are largely unknown.

4.2 Aquatic populations

Populations of aquatic organisms are also affected by exposure to insecticides that find their way to the rivers, ponds and other water bodies either through spray drift or through runoff from agricultural fields. Anecdotal accounts of fish kills and declining fish populations due to pesticides are common, but rigorous studies on the impact that individual or mixture insecticides have on particular species are rarer. Lockhart et al. (1985) showed that malathion sprayed at 210 g/ha for mosquito control in Winnipeg caused small temporary decreases in both catch of young walleye (*Stizostedion vitreum*) per unit effort and weight gains after the first spray. A reduction in AChE activity to about 25% of pre-exposure levels was estimated as the approximate threshold for population effects on this species of fish. Similar findings were reported in Haiti after spraying with malathion to control malaria vectors (McLean et al. 1975). Based on profenofos residues and AChE activity in wild fish, populations of European carp (*Cyprinus carpio*), bony bream (*Nematalosa erebi*), and mosquitofish (*Gambusia holbrooki*) are probably reduced during the peak contamination events in rivers from cotton-growing areas in Australia (Kumar and Chapman 2001). However, impacts of OP insecticides on fish populations under field conditions are likely to be minimised by the fast hydrolysis of these compounds. In the case of pyrethroids, the presence of organic matter and suspended solids in streams and rivers reduces considerably the exposure of fish to the insecticides. For example, pulses of cypermethrin applied to transgenic soybeans in the Rolling Pampas of Argentina did not result in direct mortality of the native fish *Cnesterodon decemmaculatus*, despite residue concentrations after spraying reaching comparable levels to the 96-h LC50 for this species (0.43 µg/L). The reason was the 12-fold reduction in exposure due to sequestering of residues by water particles rich in organic content (Carriquiriborde et al. 2007). Avoidance behaviour may also explain the lack of exposure of some species in natural situations. For example, populations of the freshwater amphipod *Gammarus pulex* migrated downstream when exposed to pulses of fenvalerate and parathion-methyl in agricultural streams, thus avoiding direct mortality (Schulz and Liess 1999). Similar findings were reported for populations of macro-crustaceans (*Hyalella curvispina* and *Macrobrachium borelli*) in two streams of the Argentine pampa exposed to chlorpyrifos, endosulfan and α-cypermethrin: mortality was high during the peak pulses, but declined further downstream due to migration (Jergentz et al. 2004). Populations of ostracods in rice fields are reduced significantly when treated with carbufuran, endosulfan or imidacloprid applied at their recommended field rates. Because zooplankton and epibenthic crustaceans are very sensitive to pyrethroid, carbamate, OC and OP insecticides (Table 2), they can be used as sentinel species to evaluate impacts of these chemicals. Pulse exposures of insecticides eliminate or reduce most populations of zooplankton species, but their recovery usually takes place within 15 days to 1-2 months (van den Brink et al. 1996). Recoveries depend on the residue levels in water, intraspecific competition for food and external predation pressures, as well as the presence of refuges that protect from exposure.

As with terrestrial populations, aquatic organisms are more likely to be affected in their growth and reproductive ability. For example, while the LC50 of fipronil to the estuarine copepod *Amphiascus tenuiremis* is 6.8 µg/L, concentrations of this insecticide in water at 0.22

μg/L reduced female egg extrusion by 71%, whereas 0.42 μg/L almost eliminated reproduction (94% failure) (Chandler et al. 2004). Extinction of *Daphnia pulex* populations occurred at concentrations 80 μg/L of fipronil, which is just above the NOEL threshold but higher than predicted environmental concentrations of this insecticide when applied for fruit fly control (Stark and Vargas 2005). Sublethal concentrations of fipronil and several pyrethroids up to 4.3 times lower than the LC50 for some compounds result also in growth inhibition of *Chironomus sp.* larvae. Given the life cycle of these organisms, lack of emergence into adult midges leads to ecological impacts similar to mortality, since their populations became reproductively non viable. Indeed, the zooplankton species *Daphnia pulex* went to extinction after exposure to a Neemix concentration of 0.45 mg/L (azadirachtin) even though the LC50 is 0.68 mg/L. This is because the number of offspring per surviving female declined to the point that no reproduction occurred at the sublethal concentrations (Stark 2001). Similar outcomes were observed with exposure of the copepod *Eurytemora affinis* to dieldrin, which resulted in no population growth at 10 μg/L whereas the LC50 is 23 μg/L, as well as populations of *Daphnia magna* and *D. pulex* exposed to spinosad, and *Moina macrocopa* exposed to the insect growth regulator methoprene. Pulses of esfenvalerate at concentrations in the range 0.1-0.6 μg/L can negatively affect *Gammarus pulex* survival, pairing behaviour, and reproduction even 2 weeks after the initial exposure. These amphipods are very sensitive to esfenvalerate, and exposure to 0.05 μg/L for 1 h led to immediate disruption of reproducing pairs, release of eggs or offspring from the brood pouch and, following transfer to clean water, subsequent delays in pair formation and reproduction (Cold and Forbes 2004).

Apart from direct impacts on survival and reproduction, cypermethrin appears to affect the swimming ability of *Daphnia magna* exposed to typical sublethal concentrations (0.05 and 0.6 μg/L) occurring in freshwater systems after its application. Inevitably, impaired swimming ability results in significant feeding reduction of the waterfleas. The European seabass (*Dicentrarchus labrax*) exposed to fenitrothion experienced similar effects. Other sublethal effects observed in fish include abnormal structure of the testis of bluegill (*Lepomis macrochirus*) exposed to 60 μg/L of diazinon (Dutta and Meijer 2003) and vertebral malformation in medaka fry exposed to 5-10 mg/L of carbaryl (Kashiwada et al. 2008). The high toxicity of endosulfan to adult frogs and tadpoles of various species is not mitigated by increases in water temperature in the subtropical and tropical regions where this insecticide is still applied; on the contrary, it seems that sublethal exposures (0.8 μg/L) of *Litoria citropa* tadpoles to this insecticide result in higher subsequent vulnerability to predation by dragonfly larvae (Broomhall 2002).

5. Impacts on terrestrial communities and ecosystems

Community impacts result from both direct effects on populations and indirect effects caused by the removal of prey species and/or competition from other species in the ecosystem. They have mainly been studied in mesocosms and microcosms, as these small-scale ecosystems, comprised of assemblages of a few taxa, allow monitoring under controlled experimental conditions. Impacts of insecticides on these communities affect the whole ecosystem, often resulting in functional changes that depend on the specific structure of all integrating communities. Thus, concerns over the insidious effects of pesticides operating through the disruption of the food chain structure are justified.

5.1 Soil communities

Impacts of insecticides on micro-organisms are mixed, because negative effects on one group may favour another group. For example, chlorpyrifos altered temporarily the structure of saprophytic communities of treated groundnut fields in India: the bacterial communities were inhibited for two months while the fungi communities increased concomitantly. The impact of quinalphos was similar, except that this OP insecticide also reduced the fungi in the soil. Soil protozoa can also be disturbed by insecticide applications, as their populations do not usually recover within 60 days (Foissner 1997). However, carbofuran can foster the nitrogen-fixing bacteria associated with the rice rhizosphere, with populations of *Azospirillum* sp., *Azotobacter* sp. and anaerobic nitrogen-fixing bacteria increasing progressively with a successive number of applications.

Spray drift of chlorpyrifos and azinphos-methyl applied to orchards in South Africa reduced significantly the earthworm communities in neighbouring areas, even if soil concentrations of these OPs were very low: 0.2-2.7 mg/kg and 1.6-9.8 mg/kg, respectively (Reinecke and Reinecke 2007). Phorate can also foster enchytraeid worms indirectly by eliminating their predators. In flooded soils such as rice fields, insecticides reduced populations of aquatic oligochaetes in the soil, although no significant long-term effects are observed. Some long-term studies have shown that insecticide-treated fields had no ecologically significant impacts in earthworm communities when compared to untreated fields, the differences being largely consistent with the expected effects of climate, soil types, crop types and cultivation practices.

Since the early days of pesticide usage it was realised that OC insecticides had negative or mixed effects on soil microfauna. The suppression of springtails (Collembola), saprophagous mites, symphilids and paurapods (Myriapoda) by insecticides is of concern. Paurapods seem to be most susceptible to OCs, and some communities can be completely eliminated by OP insecticides. Symphylids feed on plant rootlets, and because they live in the deep soil layers are less affected by treatments of OC and pyrethroid insecticides; however, systemic OP insecticides and fumigants can cause serious reductions in all taxa (see review in Edwards and Thompson 1973). Negative impacts of OP and carbamate insecticides on these tiny animals of the soil have also been observed, although the altered structure of their communities in soil microcosms treated with dimethoate did not disrupt the nutrient dynamics. Millipedes are more tolerant to broad-spectrum insecticides, but their populations declined over the years in cabbage plots treated with DDT, because residues of this persistent OC accumulated in their bodies, thus reducing their reproductive capacities (Brown 1978).

Mites are the most numerous arthropods in soil, with many of them being predators or saprophytic while some *Tetranychus* species are crop pests. Predatory mites are more affected when crops are treated with OC, OP and carbamate insecticides (Edwards and Thompson 1973). Although mite populations recover within six weeks to a few months, the elimination of the most susceptible Gamasina predacious mites by a single application of aldicarb at 25 kg/ha resulted in a different community structure developing in the subsequent four years (Koehler 1992). Even natural insecticides like neem extracts (azadirachtin) are more detrimental to oribatid mites than other mites and spiders. A commonly observed outcome of insecticide application is the release of prey species due to reduced predatory pressure. For example, springtails usually increase in numbers when fields are treated with recommended doses of OC and pyrethroid insecticides, since the mites that prey on them are more susceptible than their prey (Badji et al. 2007). However, chlorpyrifos decimates springtail populations and changes the structure of their

communities in soil, which take more than a year to recover. Ground spiders were also significantly reduced in British upland grasslands treated with chlorpyrifos (1.5 L/ha) even if their diversity remained similar to the non-treated plots. Consequently, one collembolan species, *Ceratophysella denticulate*, increased and dominated (>95%) the community due to lack of predatory mites and competition with other springtails (Fountain et al. 2007).
Non-target communities of ground arthropods (e.g. carabid and staphylinid beetles, ants, earwigs, centipedes and spiders) are usually decimated when broad-spectrum insecticides are aerially sprayed to agricultural fields, orchards or to control locust outbreaks. For example, more than 75% of springtails, ants and Tenebrionidae beetles were eliminated after spraying with fenitrothion in Madagascar, but most populations recovered within a year. A combination of fenitrothion and esfenvalerate did not affect as many arthropods, although spiders were more affected and springtails did not recover in one year (Peveling et al. 1999). Ants appear to be the most sensitive non-target taxon to sprays of AChE inhibitors (carbaryl, malathion) and the IGR diflubenzuron. The IGR triflumuron, by contrast, controlled the locust but had little impacts on most arthropod communities with the exception of some butterflies, Hymenoptera predators and parasitoids. The impact of modern insecticides on the latter taxa can be a concern in IPM programs aimed at preserving the biocontrol potential of parasitoids. Thus, spinosad has little effect on most predatory insects, but 78% of laboratory studies and 86% of field studies show its harmful impact on hymenopteran parasitoids through sublethal effects that include loss of reproductive capacity, reduced longevity, etc, which lead to the decline of their populations (Williams et al. 2003). Ground-dwelling carabid and staphylinid beetles make up about 75% of the predaceous and/or parasitic insects that control many crop and horticultural pests. Most OC insecticides reduce their populations and allow very slow recovery afterwards, whereas endosulfan at 1 kg/ha appears not to cause major impacts on these arthropods (Wiktelius et al. 1999). The impact of AChE inhibitors on carabid populations ranges widely among species, with carbaryl at 2.26 kg/ha in corn plantations having little impact (Whitford and Showers 1987). Pyrethroids and imidacloprid at recommended rates have also minimal impacts on these ground communities in spite of their high toxicity to insects (Sánchez-Bayo et al. 2007; Wiktelius et al. 1999).
Treatment of livestock with pour-on insecticides to control ectoparasites may have an unfortunate drawback: residues of the active compounds used (e.g. famphur, cypermethrin, spinosad, cyromazine, avermectins, fluazuron, etc.) are usually excreted and concentrated in the dung, thus killing fly maggots and dung beetles that recycle the cowpats. This causes structural changes of the saprophytic community through a reduction of species diversity and increase in dominance of less susceptible species. For example, emergence of the dung beetle *Liatongus minutus* and eight species of flies from cowpats in the first two weeks following ivermectin treatment at normal rates (0.5 mg/kg body weight) was significantly reduced, while Ceratopogonidae and Psychodidae species prospered (Iwasa et al. 2005). These impacts can occur while lethal levels of residues persist in the dung – usually 1-3 weeks for most pyrethroids and avermectins in cowpats (Krüger and Scholtz 1997), but shorter times in sheep dung. However, IRG like fluazuron and methoprene appear to have no such effects at normal rates of treatment (Niño et al. 2009).

5.2 Arthropod communities of vegetation

As might be expected, the highest impacts occur in communities of arthropods of the crop and surrounding areas. Since the early years of the Green Revolution entomologists realized the limitations of using insecticides that killed over 95% of the insect pest as well as many

non-target and beneficial insects. Indeed, elimination of natural predators usually results in the rebound of pest populations, and sometimes even in the creation of new pests. For example, carbofuran and chlorpyrifos applied to maize crops in Nicaragua reduced the foraging activity of predatory ants, thus resulting in the rebound of the noctuid *Spodoptera frugiperda* and the cicadellid *Dalbulus maidis* pest populations (Perfecto 1990).

Predatory arthropods keep the populations of insect pests in check. Ladybird beetles, dragonflies, earwigs, some ants and crab spiders predate on eggs of pest species: bollworm (*Heliothis* sp.) egg predation can be as high as 30-37% per day in cotton crops not treated with insecticides. Among the insecticides applied to tea plantations in India, pyrethroids and ethion suppressed populations of predatory *Syrphis* sp. and the ladybird *Coccinella septempunctata* for over a week, endosulfan only affected the ladybirds, whereas neem and Bt formulations did not impact on the predators (Sharma and Kashyap 2002). Testing the susceptibility of natural predators to insecticides has become a necessity in many developing countries. It appears that pyrethroids are harder on predators than OP and OC insecticides, perhaps because insects in all larval stages are very susceptible to these kind of insecticides. Parasitic Hymenoptera also play an essential part in controlling numerous pest larvae. A recent review of 39 ecosystems found that agrochemical pollutants negatively affect these parasitoids in 46% of cases (Butler et al. 2009), with persistent and systemic insecticides (e.g. cartap and imidacloprid) having the greatest impacts. However, predatory arthropods are less susceptible than parasitoids and more variable in response to pesticides, with some predatory species being very tolerant to pesticides (e.g. the spider *Lycosa pseudoannulata*, the coccinellid *Cryptolaemus montrouzieri*, and the lacewing *Chrysopa carnea*).

Early insecticide impacts on non-target arthropod communities were reported for orchards sprayed during three years with lead arsenate and nicotine. Ground-dwelling beetles, spiders and ants were reduced by 15%, and the proportion of eggs and larvae of the main apple pest – the coddling moth (*Laspeyresia pomonella*), which is parasitized by Hymenoptera species – decreased by 64-97%, allowing the moths to come back unopposed. DDT sprays helped eliminate the coddling moth, but it created new pests among leaf-rollers, woolly aphids, red-spiders and *Tetranychus* mites that surged as a consequence of the lack of predators and the suppression of parasitism. Citrus orchards sprayed with DDT to control cottony-cushion scales and mealybug pests also eliminated the predatory ladybird beetles and parasitoids – as a result, pest numbers not only did not decrease but rather surged exponentially. Because of the persistence of DDT, restoration of a normal predator-prey relationship after cessation of sprays could take up to five years (Pickett 1962). Replacement of such chemicals with other broad-spectrum insecticides did not improve the situation, as ground beetles, spiders, harvestmen, earwigs, and centipedes, which are predators of lepidopteran and homopteran pests of apple, were suppressed as well, whereas mites, slugs and snails were less affected (Epstein et al. 2000).

The annihilation of predatory and parasite arthropods in cotton, corn, rice and horticultural crops has created new community structures characterized by the absence of predator-prey relationships, one where pests species thrived for a while until the next insecticide spray decimates them, where resurgence became the norm and resistance to chemicals the final outcome (see review in Brown 1978). Pest management plans in cotton agroecosystems continued to rely on the routine and heavy use of pyrethroids, OPs, carbamates and new insecticides until the 1990s. Recently, the introduction of transgenic Bt-cotton in some countries appears to have had a positive effect on restoring the biodiversity of most predatory insects, spiders and birds in cotton fields, since insecticide applications are

reduced 50% or more (Wadhwa and Gill 2007). Similarly, the biodiversity of arthropods in Bt-corn crops is much higher than in fields treated with pyrethroids like λ-cyhalothrin, which can reduce the natural enemy community by 21-48% in a single application or by 33-70% in five applications (Rose and Dively 2007). The only indirect effect of Bt crops appears to be a reduction in parasitoids, as the Bt toxins tend to eliminate the host Lepidoptera larvae. Apart from transgenic crops, IPM strategies aimed at increasing predatory populations in cotton crops include the use of supplementary food sprays together with virus applications, which can reduce insecticide use by 50% without sacrificing cotton yield and profitability. Another strategy relies on the application of IGRs such as buprofezin or pyriproxyfen, which reduce significantly the impact on predatory taxa compared to conventional treatments with broad-spectrum insecticides. Insecticide sprays on rice crops upset natural enemy control of pests such as plant hoppers (*Nilaparvata lugens*) and also create heavy selection pressure for strains of pests that can overcome previously resistant rice cultivars. Such circumstances create outbreaks of secondary pests and impair biological control of some key primary pests such as Pyralidae stem borers. Typical applications of BHC and parathion significantly decreased densities of predatory dragonflies, spiders and parasitoids, thus increasing the herbivore:predator ratio among arthropods. The insecticides imidacloprid and fipronil also change this ratio even if their main impact appears to be on midge larvae (Chironomidae). As with corn and cotton, transgenic Bt-rice can also help reduce the impact on predators, since the cry1Ab toxins do not affect *Cyrtorhinus lividipennis*, the main predator of planthoppers (Chen et al. 2007). The rich biodiversity of rice fields in tropical countries, with some 200 species of predatory arthropods, can be successfully used in IPM programs to control the 55 species of pests found in this crop.

Spiders and phytoseiid mites are important predators in all kinds of crops. Applications of OP, carbamate and pyrethroid insecticides in vineyards, orchards and other crops usually result in increases of pest *Tetranychus* mites because of reductions in the more susceptible phytoseiid predators. Chlorpyrifos and isofenphos applied to blue turf grass in Kentucky had the greatest impact (6 weeks) on predaceous mites, while effects of bendiocarb and trichlorfon were less severe and more temporary. By contrast, oribatid mites were apparently unaffected by the insecticides. The pyrethroid λ-cyhalothrin eliminated most pest and predatory mites alike in sprayed apple orchards in Canada (Li et al. 1992), whereas predatory phytoseiid mites survived well in vineyards treated with sulphur and copper but not with synthetic insecticides in South Australia (James and Whitney 1993). In experimental plots, spiders were three times less abundant in apple orchards treated with insecticides than in untreated ones, whereas spiders and ants populations were reduced in 53% of the corn crops treated with lindane (0.5 ka/ha) in Africa, an effect that lasted 2-3 weeks (Wiktelius et al. 1999). Lycosidae and linyphiid spider populations undergo a similar pattern – they are initially eliminated from cereal fields treated with OP insecticides, but their abundance may increase subsequently in response to rebound densities of unaffected prey like springtails.

In a few cases, however, the use of insecticides to control introduced pests can overcome the ecological damage that would follow if the pest were left unchecked. For instance, the exotic gypsy moth (*Lymantria dispar*) is invading the forested ecosystems in North America. The defoliation caused by outbreaks of this species has severe environmental impacts: death of oak trees, which results in less acorn production leading in turn to reduced populations of squirrels; outcompeting the native tiger moths (Arctiidae) for food; increasing bird nest predation as visibility increases due to lack of canopy; and replacement of oaks with maple trees, thus altering the foraging patterns of large mammals such as deer and bear. Spraying

the affected forests with Bt insecticides, which has practically no impacts on non-target species, avoids the cascade of ecological impacts caused by the moth (Thompson 2011).

5.3 Vertebrate communities

Insecticides indirectly affect insectivorous vertebrates by reducing the insect prey base available to them. For example, populations of insectivorous birds such as white-headed black chats (*Thamnolaea arnoti*) can be reduced by 74-88% when their food supply disappears after spraying with DDT for tse-tse control in Africa (Douthwaite 1992).

The best documented evidence of indirect pesticide effects on bird populations is found in the United Kingdom, where declines of the grey partridge (*Perdix perdix*) had been noticed by game hunters and ornithologists for some time – it was rightly attributed to the combined indirect effect of herbicides and insecticides that resulted in breeding failure as a consequence of chick starvation and low survival (Potts 1986). In addition, other contributing factors such as worm parasites have added to the partridge demise. Since insecticides are routinely sprayed on cereal and other crops everywhere they have been indirectly affecting the food supply of many bird populations, some of which are declining in European countries and North America (Donald et al. 2001; Peakall and Carter 1997). Declining bird species (e.g. song thrush, spotted flycatcher, etc.) are not associated with particular foods, but with overall reductions in abundance and diversity of plants, seeds and insects as a result of intensive agriculture and loss of habitat. Although such declines are primarily driven by herbicide use and the switch from spring-sown to autumn-sown cereals, both of which have massively reduced the food supplies of granivorous birds (Newton 2004), insecticides account for a large part in those impacts, as most phytophagous birds supplement the diet of their offspring with insects. During the bird breeding season, grasshoppers, sawflies, spiders, leaf-beetles, weevils, butterflies/moths and their larvae, aphids, and crane-flies and their larvae are important foods for insectivorous, omnivorous and granivorous birds. The first four insect taxa are associated with the diet of most declining bird species and are also very susceptible to most insecticides even if birds may be tolerant. Failure to rear their young subsequently results in lower reproductive rates that can bring demise to some species. Recovery of plant and insect densities can be achieved in a few years once the intensive management practices are abandoned, offering hope for the recovery of birds as well.

It is reasonable to assume a similar fate in small insectivorous mammal and reptile populations, but at present evidence from field studies on these animal taxa is scarce (reviewed by Story and Cox 2001). For example, fenitrothion sprayed to control an outbreak of jack pine budworm (*Choristoneura pinus*) in pine plantations in Canada reduced populations of shrews indirectly due to lack of their available arthropod prey, whereas aminocarb and Bt did not have any impacts on small mammals (Innes and Bendell 1988). Relationships among mammal species may also be indirectly affected as a consequence of food depletion. Thus, while diazinon and carbaryl applications on prairie grasslands directly affected cotton rats (*Sigmodon hispidus*) because they consume contaminated prey insects, other mammals in the community such as prairie voles (*Microtus ochrogaster*) and house mice (*Mus musculus*) increased their numbers perhaps due to lack of competition with the rats. When the grassland was sprayed with dimethoate, house mice decreased while prairie voles increased and deer mice (*Peromyscus maniculatus*) remained at the same density (Barrett and Darnell 1967).

6. Impacts on aquatic ecosystems

Residues of insecticides applied to agriculture find their way to the streams, rivers, ponds, lakes, and ultimately to estuaries and the sea mainly through runoff. Amounts discharged vary among chemicals, but usually not more than 1-6% of the applied amount goes into the water systems in one season. In addition, spray droplets can fall directly onto the water surfaces of agricultural ditches, streams and ponds near the fields, thus causing unintended damage to their aquatic communities because neurotoxic insecticides are particularly toxic to fish and crustaceans (Table 2). The adoption of buffer strips and vegetated barriers to stop drift contamination of water bodies is a recommended management practice in many developed countries, one that can reduce drift by 95%.

6.1 Invertebrates

Insecticides can eliminate entire populations of zooplankton species even at very small concentrations in water and sediments. For instance, residues of imidacloprid at about 1 μg/L in experimental rice paddies wiped out several ostracod and cladoceran species, as well as one species of *Chironomus* larvae (Sánchez-Bayo and Goka 2006). Chlorpyrifos decimates zooplankton communities at concentrations above 0.1 μg/L (López-Mancisidor et al. 2008), introducing indirect effects due to shifts in competition and predation between populations. Thus, a sudden collapse in cladoceran populations usually results in rotifers increasing as a consequence of reduced competition for food, but only when other predatory pressures on the rotifers are also suppressed by the insecticides. This is because rotifers are usually more tolerant of OP and carbamate insecticides than the cladocerans. Alternatively, when the copepods in the community are eliminated by 10 μg/L of neem (azadirachtin a.i.) the cladocerans increase concomitantly (Kreutzweiser et al. 2004). The most common indirect effect of the insecticide-impacted zooplankton communities is the surge in algae blooms due to reduced grazing pressure by micro-crustaceans in the ecosystem. When primary producers such as algae grow out of control due to the elimination of herbivorous zooplankton, the pH and oxygen concentration of the waters increase. However, this effect, does not last long because the organisms killed by the insecticide are then decomposed by fungi and bacteria, thus counteracting to some extent those chemical changes in the water (Schäfer et al. 2011). These community and ecological effects have been observed with many insecticides, and trials on Sahelian ponds have shown that pyrethroids, carbamate and OP insecticides have greater impacts on the zooplankton communities (Anostraca and Branchiopoda) and aquatic insects (Notonectidae) than diflubenzuron and fipronil (Lahr 1998).

Apart from zooplankton, other invertebrates such as aquatic insects, their larvae (nauplii), and worms are affected by insecticide drift or in runoff. Caddisflies, mayflies and stoneflies nauplii, amphipods, isopods and shrimps are decomposers of plant litter and dead organisms, whereas dragonflies, Dytiscidae and Hydrophilidae beetles, backswimmers, striders, etc. are predators: all of them are very sensitive to neurotoxic insecticides and IGR (Table 2). Several species of invertebrate communities such as these were eliminated when surface runoff after rainfall resulted in contamination with parathion-ethyl, lindane and fenvalerate in an agricultural catchment in Germany (Liess and Schulz 1999). Often, mobile species like the amphipod *Gammarus* sp. move to less contaminated sections to avoid direct insecticidal effects. Elimination of decomposers from the aquatic communities due to insecticides resulted in a three- to five-fold decrease in leaf-litter decomposition in 16 French

streams (Schäfer et al. 2007). Such impacts can affect several kilometres of river downstream, because many aquatic organisms, including fish, rely on particulate organic matter input from upstream sections. Shrimps, in particular, are very sensitive to pyrethroids and fipronil. For instance, populations of grass shrimp (*Palaemonetes pugio*) were eliminated from estuarine mesocosms treated at 5 mg/L fipronil, and reduced 60% at 0.35 mg/L, whereas oysters, clams or fish (*Cyprinidon variegatus*) in the same mesocosms were not affected at all (Wirth et al. 2004). Among the decomposers and shredders of the benthic communities, midge larvae and amphipods are very sensitive to OP insecticides and were severely affected in wetlands treated with phorate (1.2-4.8 kg/ha), whereas leeches, snails, worms and ostracods were hardly affected (Dieter et al. 1996). For this reason, midges and amphipods are preferred sentinel species for the detection of pesticide contamination in rivers, estuaries and coastal waters. Recovery of the impacted communities can take from 1-2 months in the case of zooplankton (van den Brink et al. 1996) and aquatic larvae (Dieter et al. 1996), to several months in the case of macroinvertebrates, and for some species recovery may not happen if runoff insecticide inputs keep occurring over the years (Liess and Schulz 1999). Apart from the sensitivity of certain taxa to particular pesticides, recovery of the community depends to some extent on the climatic conditions and the agricultural practices. Thus, in tropical agriculture, where high quantities of insecticides are applied, the toxic levels in ditches and rivers may be offset by the faster dissipation that such compounds undergo in the warm and humid conditions of the tropics. After some 20 years of treatments with a diverse array of larvicides (temephos, pyraclofos, carbosulfan, permethrin, Bt, etc.) for the control of the blackfly (*Simulium damnosum*) –the main vector of blindness disease-, the aquatic communities of African rivers revealed only temporary changes in structure that did not compromise the overall functionality of the aquatic ecosystems (Crosa et al. 1998).

6.2 Vertebrates

Fish are very susceptible to pyrethroid insecticides (Table 2). When esfenvalerate drift or runoff residues reached ponds containing bluegill (*Lepomis macrochirus*), the small fry (<2 cm) went missing in ponds with the highest concentrations (2 μg/L) (Webber et al. 1992). Most pyrethroid impacts, however, are transient and fish recover quickly after the residues dissipate. Impacts on fish communities by insecticides are more likely the result of indirect effects through the food chain, but studies at the community level are rare. Some understanding has come from insecticide spills into rivers. Thus, a spill of toxaphene from a cattle dip tank in August 1978 affected most fish species in a 26 km stretch of the Hluhluwe River (Natal, South Africa). Although most fish reappeared after a year, due to migration from upstream tributaries, their populations were very low. The bioaccumulation of this OC insecticide was apparent in fish-eating birds such as herons and raptors, some of which were endangered (Brooks and Gardner 1981). However, repeated application of insecticides in African rivers for the control of onchocerciasis (see above) does not show any clear trend in the fish populations, although some species have been reduced temporarily.

The fact that many amphibian population declines occur in intensive agricultural areas has alerted some researchers. Some blame the combination of indirect effects from insecticides and herbicides, which introduce a cascade of events affecting negatively the feeding and growth of tadpoles plus sublethal effects involving stress and parasite infections. Endocrine disruption affecting larval growth and development of tadpoles has also been observed with mixtures of insecticides and herbicides in mesocosms (Hayes et al. 2006). These impacts together with other intensive farming practices, such as the use of fertilizers, may be in part

responsible for declines in some amphibian species (reviewed in Mann et al. 2009). Nevertheless, tadpoles of southern leopard frogs (*Rana sphenocephala*) increased in numbers when carbaryl reduced their predators in a mesocosms; the unforseen effect of such a boost was increased intra-specific competition for the periphyton, which resulted in smaller metamorphs. Similar impacts were observed with malathion on six amphibian species in more complex aquatic communities (Relyea and Hoverman 2008). However, pesticide-treated rice paddies continue to be a valuable haven for many species of frogs, since herons are not attracted to conventional fields because they have less foraging value than organic ones.

Studies on insecticide impacts in communities of aquatic reptiles, birds and mammals are non-existent. All we know is that residues of persistent OC insecticides are found in large marine mammals throughout the world, particularly whales, dolphins and seals in the Mediterranean regions and the west coast of North America (Aguilar et al. 2002). Recent studies on loggerhead sea turtles (*Caretta caretta*) confirm the widespread contamination of the oceans with DDT, toxaphene, mirex, chlordanes, lindane, dieldrin and other persistent organic pollutants (Alava et al. 2011), but the impacts these residues may have on the ecology of these animals is only hypothetical.

7. Risk assessment

Whilst the term ''hazard'' indicates the existence of potential harm, ''risk'' refers to the probability of harm occurring. Risk assessments aim at preventing or at least minimising such impacts based on our current knowledge of the hazardous pesticides used in agriculture, forestry and urban environments. Here I described some of the actual risks posed by commonly used insecticides, either in real field situations or on artificial, simulated ecosystems. And yet, despite this evidence, current methods to assess the risk of pesticides are still inadequate because the overwhelming majority of the models use simple LD50 or LC50 estimates. As we have seen, this type of information is limited, since the sublethal effects of insecticides can affect populations at concentrations far lower than those determined in acute toxicity tests. For example, Giddings et al. (2001) showed that the LOEC values for the pyrethroids cypermethrin and esfenvalerate in mesocosms were one tenth of those derived in laboratory experiments. In addition, indirect effects on other species of the community are almost unpredictable since we do not have models to describe them.

A distinction must be made, however, between the two types of risk assessment currently in use. Firstly, ecological risk assessment methods that aim at protecting the ecosystem, its communities and species have improved remarkably in the last decade. Species sensitivity distributions (SSD) take into account either the lethal or the no-observed effect levels of all species tested so far, allowing estimation of the median hazard levels for a given percentile of species (e.g. HC5 for effect on 5% of species, equivalent to 95% species protection) (Posthuma et al. 2002). Although the accuracy of these predictions relies entirely on the number of species tested and the taxa covered in the distribution, the SSD model has been validated for both aquatic and terrestrial communities. Models based on SSD are currently used to set water quality and sediment guidelines in many countries.

The second type of risk assessment is for regulatory purposes in agriculture or forestry. Here the aim is to minimise the impact on ecosystems to levels accepted by the community, which is not necessarily the same as protecting the integrity of the ecosystems. As a

consequence, the registration process considers several 'tiers' of assessment. Testing for lethal dose or concentration in a few species representative of a community of organisms (e.g. *Daphnia*, trout or another fish, honeybee, rat and quail or duck) is usually the norm for the first tier of the assessment. Higher tiers may involve mesocosm or field studies, but only when the data for the first tier appears to be insufficient to prove the 'safety' of the chemical. Safety, of course, may apply to some organisms but not others, as discussed above (see Table 2). Although recommendations and regulations for insecticide assessment, registration and re-evaluation are now in place in most of the developed world (reviewed by Greig-Smith 1992), there are many discrepancies between the regulations of various countries even for the same chemical (Devine and Furlong 2007). Apart from persistent OC insecticides, most of which are banned in developed countries, any other insecticide is allowed in practice as long as certain precautions and management options are put in place. Pesticide labels are supposed to take care of this, but this assumes that all farmers have a good level of literacy, which is far from the reality. The problem lies in the implementation of those precautionary measures among the farming communities, more so in countries with little or no legislative power in this matter. Not surprisingly, developing countries are lagging behind in these matters.

Since the bulk of insecticides is being used in developing countries, most of which are located in the tropical or subtropical regions of the world, insecticide risk assessment should be a priority in such regions. Using tropical taxa for the regulatory testing has been suggested, arguing that their species may differ in susceptibility to pesticides. However, most tropical species do not differ in sensitivity from their temperate counterparts (Daam et al. 2009; Kwok et al. 2007). As for the higher dissipation rates of pesticides in the tropical regions, which may minimise exposure to organisms, most chemicals appear to pose a similar risk as in temperate regions because the increasing losses by degradation or volatilisation are usually counterbalanced by greater desorption and movement of residues into the aquatic environment (Sanchez-Bayo and Hyne 2011).

8. Insecticides in sustainable agriculture

There is no doubt that insecticides help produce higher crop yields because they reduce substantially the damage inflicted by insects and other pests (Pimentel 2005). However, the ecological cost of conventional agricultural practices is also evident, even if the extent of the disturbances they cause varies markedly among compounds. Except for a few accidental cases, most of the impacts described in this chapter occur when insecticides are used as recommended. Impacts range from short-term imbalances in the planktonic and invertebrate communities to long-term reduction in the reproductive ability of some bird and fish species, which may lead to the extinction of the populations affected. The severity of the impacts depends not only on the toxicity of the compounds but also on unforeseen sublethal and indirect effects they may cause. Physico-chemical characteristics of the compounds are equally important, since they determine their persistence and bioaccumulation properties in organisms, thus being intimately associated with the exposure and long-term effects in the ecosystems. It has also been shown that such ecological damage is widespread throughout the world, although it is more prevalent in agricultural areas of temperate and tropical regions. On a positive note, the capacity of the ecosystems to recover from insecticide pulses is remarkable. Even the worst cases of

pesticide contamination, such as the storehouse spill in the Rhine in November 1986, can return to normality after one or two years (Capel et al. 1988).

Awareness of the impacts that insecticides are having in our world may help introduce management practices with the aim of reducing and mitigating those impacts. Historically, such awareness has prompted the search for new pest control technologies such as biological control, pheromone traps, attractants and others included under the current IPM practices. It has also helped develop on-site mitigating technologies such as phytoremediation. Certainly, IPM has not achieved a reduction in overall pesticide usage, as it was originally intended, even in areas where that concept is very popular (e.g., the UK and California), but at least has kept insecticide use static during a period of increasing agricultural intensification (Devine and Furlong 2007). Moreover, IPM practices are only compatible with selective and non-persistent insecticides that do not affect predatory and parasitic organisms. Thus, it excludes most of the highly toxic, broad-spectrum and persistent insecticides, which are the ones that cause most damage to the environment. In addition, IPM practices are economically more affordable because of the low insecticide inputs they require, and yield profit margins about 2% higher than conventional agriculture (Young et al. 2001). Hence, the adoption of IPM in poor countries is imperative, not only to avoid the pollution and ecological degradation of their environment but most importantly to help poor farmers develop a sustainable agriculture which does not depend on costly chemical inputs.

Finally, ecological damage usually cannot be measured in economic terms, unless they involved losses of game, livestock, soil fertility or any other issue with economic value. This only brings in confusion among the public, farmers, stakeholders and politicians, who see the reality from different points of view and, therefore, hold very different opinions on these matters. Thus, the decisions following specific risk assessments of insecticides are always subjective, since there is no metric that can compare the ecological consequences of using a given insecticide to the economic profits it may bring to a particular farming community or a country.

9. Conclusion

The environmental pollution and a subtle undermining of the ecosystems brought about by the constant use of pesticides should not be dismissed as trivial. Ultimately, we all depend on the services that the environment provides for our own health and food production. Even if natural ecosystems are resilient and populations of organisms can recover relatively quickly, the constant use of insecticides in agricultural lands and forests year after year is reducing the food supplies for many vertebrate species, i.e. birds and possibly frogs, lizards and small insectivorous mammals. Thus, the increase in agricultural productivity we enjoy is paid for by a reduction of aquatic and terrestrial communities of organisms at a global scale.

10. References

Aguilar, A., Borrell, A. & Reijnders, P.J.H. (2002). Geographical and temporal variation in levels of organochlorine contaminants in marine mammals. *Mar. Environ. Res.* 53(5):425-452.

Alava, J.J., Keller, J.M., Wyneken, J., Crowder, L., Scott, G. & Kucklick, J.R. (2011). Geographical variation of persistent organic pollutants in eggs of threatened loggerhead sea turtles (*Caretta caretta*) from southeastern United States. *Environ. Toxicol. Chem.* 30(7):1677-1688.

Badji, C.A., Guedes, R.N.C., Silva, A.A., Correa, A.S., Queiroz, M.E.L.R. & Michereff Filho, M. (2007). Non-target impact of deltamethrin on soil arthropods of maize fields under conventional and no-tillage cultivation. *J. Appl. Entomol.* 131(1):50-58.

Barrett, G.W. (1988). Effects of Sevin on small mammal populations in agricultural and oil field ecosystems. *J. Mammal.* 69:731-739.

Barrett, G.W. & Darnell, R.M. (1967). Effects of dimethoate on small mammal populations. *Am. Midland Nat.* 77:164-175.

Bishop, C.A., Boermans, H.J., Ng, P., Campbell, G.D. & Struger, J. (1998). Health of tree swallows (*Tachycineta bicolor*) nesting in pesticide-sprayed apple orchards in Ontario, Canada. II. Sex and thyroid hormone concentrations in testes development. *J. Toxicol. Environ. Health A* 55(8):561-581.

Bonmatin, J.M., Marchand, P.A., Charvet, R., Moineau, I., Bengsch, E.R. & Colin, M.E. (2005). Quantification of imidacloprid uptake in maize crops. *J. Agric. Food Chem.* 53(13):5336-5341.

Brooks, P. & Gardner, B. (1981). Effect of cattle dip containing toxaphene on the fauna of a South African river. *J. Limnol. Soc. South Afr.* 6(2):113-118.

Broomhall, S.D. (2002). The effects of endosulfan and variable water temperature on survivorship and subsequent vulnerability to predation in *Litoria citropa* tadpoles *Aquat. Toxicol.* 61:243-250.

Brown, A.W.A. (1978). *Ecology of Pesticides.* John Wiley & Sons, Inc., 0-471-10790-5, New York.

Butler, C.D., Beckage, N.E. & Trumble, J.T. (2009). Effects of terrestrial pollutants on insect parasitoids. *Environ. Toxicol. Chem.* 28(6):1111-1119.

Capel, P.D., Giger, W., Reichert, P. & Wanner, O. (1988). Accidental input of pesticides into the Rhine River. *Environ. Sci. Technol.* 22(9):992-997.

Capowiez, Y., Bastardie, F. & Costagliola, G. (2006). Sublethal effects of imidacloprid on the burrowing behaviour of two earthworm species: modifications of the 3D burrow systems in artificial cores and consequences on gas diffusion in soil. *Soil Biol. Biochem.* 38(2):285-293.

Carriquiriborde, P., Diaz, J., Mugni, H., Bonetto, C. & Ronco, A.E. (2007). Impact of cypermethrin on stream fish populations under field-use in biotech-soybean production. *Chemosphere* 68(4):613-621.

Chandler, G.T., Cary, T.L., Volz, D.C., Walse, S.S., Ferry, J.L. & Klosterha, S.L. (2004). Fipronil effects on estuarine copepod (*Amphiascus tenuiremis*) development, fertility, and reproduction: a rapid life-cycle assay in 96-well microplate format. *Environ. Toxicol. Chem.* 23(1):117-124.

Chen, M., Liu, Z.-C., Ye, G.-y., Shen, Z.-c., Hu, C., Peng, Y.-f., Altosaar, I. & Shelton, A.M. (2007). Impacts of transgenic cry1Ab rice on non-target planthoppers and their main predator *Cyrtorhinus lividipennis* (Hemiptera : Miridae) - A case study of the compatibility of Bt rice with biological control. *Biol. Control* 42(2):242-250.

Clark, D.R. (2001). DDT and the decline of free-tailed bats (*Tadarida brasiliensis*) at Carlsbad Cavern, New Mexico *Arch. Environ. Contam. Toxicol.* 40(4):537-543.

Cold, A. & Forbes, V.E. (2004). Consequences of a short pulse of pesticide exposure for survival and reproduction of *Gammarus pulex*. *Aquat. Toxicol.* 67(3):287-299.

Cooke, A.S. (1973). Shell thinning in avian eggs by environmental pollutants. *Environ. Pollut.* 4:85-152.

Cox, R.L. & Wilson, W.T. (1984). Effects of permethrin on the behavior of individually tagged honey bees, *Apis mellifera* L. (Hymenoptera: Apidae). *Environ. Entomol.* 13(2):375-378.

Crosa, G., Yameogo, L., Calamari, D. & Hougard, J.M. (1998). Long term quantitative ecological assessment of insecticides treatments in four African Rivers: a methodological approach. *Chemosphere* 37:2847-2858.

Custer, T.W., Custer, C.M., Hines, R.K., Gutreuter, S., Stromborg, K.L., Allen, P.D. & Melancon, M.J. (1999). Organochlorine contaminants and reproductive success of double-crested cormorants from Green Bay, Wisconsin, USA. *Environ. Toxicol. Chem.* 18(6):1209-1217.

Custer, T.W., Hensler, G.L. & Kaiser, T.E. (1983). Clutch size, reproductive success, and organochlorine contaminants in Atlantic coast black-crowned night-herons. *Auk* 100:699-710.

Daam, M.A., Satapornvanit, K., van den Brink, P.J. & Nogueira, A.J.A. (2009). Sensitivity of macroinvertebrates to carbendazim under semi-field conditions in Thailand: implications for the use of temperate toxicity data in a tropical risk assessment of fungicides. *Chemosphere* 74(9):1187-1194.

Daryanto, I. (1998). Pesticide management policy in Indonesia. In: *Seeking Agricultural Produce Free of Pesticide Residues*, Kennedy, I.R. et al., editors, pp. 31-36, Australian Centre for International Agricultural Research (ACIAR), Canberra

Devine, G.J. & Furlong, M.J. (2007). Insecticide use: contexts and ecological consequences. *Agric. Human Values* 24:281-306.

Dieter, C.D., Duffy, W.G. & Flake, L.D. (1996). The effect of phorate on wetland macroinvertebrates. *Environ. Toxicol. Chem.* 15(3):308-312.

Dittbrenner, N., Triebskorn, R., Moser, I. & Capowiez, Y. (2010). Physiological and behavioural effects of imidacloprid on two ecologically relevant earthworm species (*Lumbricus terrestris* and *Aporrectodea caliginosa*). *Ecotoxicology* 19:1567-1573.

Donald, P.F., Green, R.E. & Heath, M.F. (2001). Agricultural intensification and the collapse of Europe's farmland bird populations. *Proc. R Soc. B.* 268(1462):25-29.

Douthwaite, R.J. (1992). Effects of DDT treatments applied for tsetse fly control on white-headed black chat (*Thamnolaea arnoti*) populations in Zimbabwe. Part I: Population changes. *Ecotoxicology* 1(1):17-30.

Dutta, H.M. & Meijer, H.J.M. (2003). Sublethal effects of diazinon on the structure of the testis of bluegill, *Lepomis macrochirus*: a microscopic analysis. *Environ. Pollut.* 125(3):355-360.

Edwards, C.A. & Thompson, A.R. (1973). Pesticides and the soil fauna. *Residue Rev.* 45:1-79.

Elliott, J.E., Birmingham, A.L., Wilson, L.K., McAdie, M., Trudeau, S. & Mineau, P. (2008). Fonofos poisons raptors and waterfowl several months after granular application. *Environ. Toxicol. Chem.* 27(2):452-460.

Epstein, D.L., Zack, R.S., Brunner, J.F., Gut, L. & Brown, J.J. (2000). Effects of broad-spectrum insecticides on epigeal arthropod biodiversity in Pacific Northwest apple orchards *Environ. Entomol.* 29(2):340-348.

FAO (2004). The State of Food Insecurity in the World. Rome: Food and Agricultural Organisation of the United Nations. 40 p.

FAO (2010). *FAOSTAT On-line Statistical Service*. Food and Agriculture Organization of the United Nations.

Foissner, W. (1997). Protozoa as bioindicators in agroecosystems, with emphasis on farming practices, biocides, and biodiversity *Agric. Ecosyst. Environ.* 62(2-3):93-103.

Fountain, M.T., Brown, V.K., Gange, A.C., Symondson, W.O.C. & Murray, P.J. (2007). The effects of the insecticide chlorpyrifos on spider and Collembola communities. *Pedobiologia* 51(2):147-158.

George, J.L. & Stickel, W.H. (1949). Wildlife effects of DDT dust used for tick control on a Texas prairie. *Am. Midland Nat.* 42(1):228-237.

Giddings, J.M., Solomon, K.R. & Maund, S.J. (2001). Probabilistic risk assessment of cotton pyrethroids: II. Aquatic mesocosms and field studies. *Environ. Toxicol. Chem.* 20(3):660-668.

Goldstein, M., Lacher, T.J., Zaccagnini, M., Parker, M. & Hooper, M. (1999). Monitoring and assessment of Swainson's hawks in Argentina following restrictions on monocrotophos use, 1996-97. *Ecotoxicology* 8(3):215-224.

Greig-Smith, P.W. (1992). A european perspective on ecological risk assessment, illustrated by pesticide registration procedures in the United Kingdom. *Environ. Toxicol. Chem.* 11(12):1673-1689.

Greig-Smith, P.W., Thompson, H.M., Hardy, A.R., Bew, M.H., Findlay, E. & Stevenson, J.H. (1994). Incidents of poisoning of honeybees (*Apis mellifera*) by agricultural pesticides in Great Britain 1981-1991. *Crop Protection* 13:567-581.

Grue, C.E., Powell, G.V.N. & McChesney, M.J. (1982). Care of nestlings by wild female starlings exposed to an organophosphate pesticide. *J. Appl. Ecol.* 19:327-335.

Guillette, L.J., Gross, T.S., Gross, D.A., Rooney, A.A. & Percival, H.F. (1995). Gonadal steroidogenesis *in vitro* from juvenile alligators obtained from contaminated or control lakes. *Environ. Health Perspect.* 103(Suppl. 4):31-36.

Hardy, A.R., Westlake, G.E., Lloyd, G.A., Brown, P.M., Greig-Smith, P.W., Fletcher, M.R., Tarrant, K.A. & Stanley, P.I. (1993). An intensive field trial to assess hazards to birds and mammals from the use of methiocarb as a bird repellent on ripening cherries. *Ecotoxicology* 2(1):1-31.

Hayes, T.B., Case, P., Chui, S., Chung, D., Haeffele, C., Haston, K., Lee, M., Mai, V.P., Marjuoa, Y., Parker, J.et al. (2006). Pesticide mixtures, endocrine disruption and amphibian declines: are we underestimating the impact? *Environ. Health Perspect.* 114(Suppl. 1):40-50.

Hernández, M., González, L.M., Oria, J., Sánchez, R. & Arroyo, B. (2008). Influence of contamination by organochlorine pesticides and polychlorinated biphenyls on the

breeding of the Spanish imperial eagle (*Aquila adalberti*). *Environ. Toxicol. Chem.* 27(2):433-441.

Innes, D.G.L. & Bendell, J.F. (1988). The effects on small-mammal populations of aerial applications of *Bacillus thuringiensis*, fenitrothion, and Matacil® used against jack pine budworm in Ontario *Can. J. Zool.* 67(5):1318-323.

Iwasa, M., Nakamura, T., Fukaki, K. & Yamashita, N. (2005). Nontarget effects of ivermectin on coprophagous insects in Japan. *Environ. Entomol.* 34(6):1485-1492.

James, D.G. & Whitney, J. (1993). Mite populations on grapevines in south-eastern Australia: implications for biological control of grapevine mites (Acarina: Tenuipalpidae, Eriophyidae) *Exp. Appl. Acarol.* 17(4):259-270.

Jergentz, S., Pessacq, P., Mugni, H., Bonetto, C. & Schulz, R. (2004). Linking *in situ* bioassays and population dynamics of macroinvertebrates to assess agricultural contamination in streams of the Argentine pampa. *Ecotoxicol. Environ. Saf.* 59(1):133-141.

Kashiwada, S., Tatsuta, H., Kameshiro, M., Sugaya, Y., Sabo-Attwood, T., Chandler, G.T., Ferguson, P.L. & Goka, K. (2008). Stage-dependent differences in effects of carbaryl on population growth rate in Japanese medaka (*Oryzias latipes*). *Environ. Toxicol. Chem.* 27(11):2397-2402.

Koehler, H.H. (1992). The use of soil mesofauna for the judgement of chemical impact on ecosystems. *Agric. Ecosyst. Environ.* 40(1-4):193-205.

Kreutzweiser, D.P., Back, R.C., Sutton, T.M., Pangle, K.L. & Thompson, D.G. (2004). Aquatic mesocosm assessments of a neem (azadirachtin) insecticide at environmentally realistic concentrations - 2: zooplankton community responses and recovery. *Ecotoxicol. Environ. Saf.* 59(2):194-204.

Kreutzweiser, D.P., Good, K.P., Chartrand, D.T., Scarr, T.A., Holmes, S.B. & Thompson, D.G. (2008). Effects on litter-dwelling earthworms and microbial decomposition of soil-applied imidacloprid for control of wood-boring insects. *Pest Manag. Sci.* 64(2):112-118.

Krüger, K. & Scholtz, C.H. (1997). Lethal and sublethal effects of ivermectin on the dung-breeding bettles *Euoniticellus intermedius* (Reiche) and *Onitis alexis* Klug (Coleoptera, Scarabaeidae) *Agric. Ecosyst. Environ.* 61:123-131.

Kumar, A. & Chapman, J.C. (2001). Profenofos residues in wild fish from cotton-growing areas of New South Wales. *J. Environ. Qual.* 30:740-750.

Kwok, K.W., Leung, K.M., Lui, G.S., Chu, V.K., Lam, P.K., Morritt, D., Maltby, L., Brock, T.C., van den Brink, P.J., Warne, M.S.J.et al. (2007). Comparison of tropical and temperate freshwater animal species' acute sensitivities to chemicals: implications for deriving safe extrapolation factors. *Integr. Environ. Assess. Manage.* 3:49-67.

Lahr, J. (1998). An ecological assessment of the hazard of eight insecticides used in desert locust control, to invertebrates in temporary ponds in the Sahel. *Aquat. Ecol.* 32(2):153-162.

Li, S.Y., Harmsen, R. & Thistlewood, H.M.A. (1992). The effect of pyrethroid lambda-cyhalothrin applications on the spatial distribution of phytophagous and predatory mites in apple orchards *Exp. Appl. Acarol.* 15(4):259-269.

Liess, M. & Schulz, R. (1999). Linking insecticide contamination and population response in an agricultural stream. *Environ. Toxicol. Chem.* 18(9):1948-1955.

Lockhart, W.L., Metner, D.A., Ward, F.J. & Swanson, G.M. (1985). Population and cholinesterase responses in fish exposed to malathion sprays. *Pestic. Biochem. Physiol.* 24(1):12-18.

López-Mancisidor, P., Carbonell, G., Fernández, C. & Tarazona, J.V. (2008). Ecological impact of repeated applications of chlorpyrifos on zooplankton community in mesocosms under Mediterranean conditions. *Ecotoxicology* 17(8):811-825.

Luo, Y., Zang, Y., Zhong, Y. & Kong, Z. (1999). Toxicological study of two novel pesticides on earthworm *Eisenia foetida*. *Chemosphere* 39(13):2347-2356.

Mann, R.M., Hyne, R.V., Choung, C.B. & Wilson, S.P. (2009). Amphibians and agricultural chemicals: review of the risks in a complex environment. *Environ. Pollut.* 157(11):2903-2927.

Manning, T. (2005). Endocrine disrupting chemicals - A review of the state of the science. *Australas. J. Ecotoxicol.* 11(1):1-52.

Maxim, L. & Sluijs, J.P.v.d. (2007). Uncertainty: Cause or effect of stakeholders' debates? Analysis of a case study: the risk for honeybees of the insecticide Gaucho. *Sci. Total Environ.* 376:1-17.

McLean, R., Spillane, J. & Miles, J. (1975). A prospective study of the effects of ultralow volume (ULV) aerial application of malathion on epidemic *Plasmodium falciparum* malaria. 3. Ecological aspects. *Am. J. Trop. Med. Hyg.* 24(2):193-198.

Mendelssohn, H. & Paz, U. (1977). Mass mortality of birds of prey caused by Azodrin, an organophosphorus insecticide. *Biol. Conserv.* 11(3):163-170.

Mineau, P. (2003). Avian Species. In: *Encyclopedia of Agrochemicals*, Plimmer, J.R. et al., editors, pp. 1-27, John Wiley & Sons, Inc.,

Mineau, P., Fletcher, M.R., Glaser, L.C., Thomas, N.J., Brassard, C., Wilson, L.K., Elliott, J.E., Lyon, L.A., Henny, C.J., Bollinger, T.et al. (1999). Poisoning of raptors with organophosphorous and carbamate pesticides with emphasis on Canada, the United States and the United Kigdom. *J. Raptor Res.* 33(1):1-37.

Morris, R.D. (1970). The effects of endrin on *Microtus* and *Peromyscus*. I. Unenclosed field populations *Can. J. Zool.* 48(4):685-708.

Newton, I. (2004). The recent declines of farmland bird populations in Britain: an appraisal of causal factors and conservation actions. *Ibis* 146:579-600.

Niño, E.L., Sorenson, C.E., Washburn, S.P. & Watson, D.W. (2009). Effects of the insect growth regulator, methoprene, on *Onthophagus taurus* (Coleoptera: Scarabaeidae). *Environ. Entomol.* 38(2):493-498.

Pain, D.J., Gargi, R., Cunningham, A.A., Jones, A. & Prakash, V. (2004). Mortality of globally threatened Sarus cranes (*Grus antigon*) from monocrotophos poisoning in India. *Sci. Total Environ.* 326(1-3):55-61.

Peakall, D.B. & Carter, N. 1997 Decreases in farmland birds and agricultural practices: a huge ecotoxicological experiment. *Toxicol. Ecotoxicol. News*:162-163.

Perfecto, I. (1990). Indirect and direct effects in a tropical agroecosystem: the maize-pest-ant system in Nicaragua. *Ecology* 71(6):2125-2134.

Peveling, R., Rafanomezantsoa, J.-J., Razafinirina, R., Tovonkery, R. & Zafimaniry, G. (1999). Environmental impact of the locust control agents fenitrothion, fenitrothion-esfenvalerate and triflumuron on terrestrial arthropods in Madagascar. *Crop Protection* 18:659-676.

Pickett, A.D. (1962). Pesticides and the biological control of arthropod pests. *World Rev. Pest Control* 1:19-25.

Pimentel, D. (2005). Environmental and economic costs of the application of pesticides primarily in the United States. *Environ. Develop. Sustain.* 7(2):229-252.

Posthuma, L., II, G.W.S. & Traas, T.P., editors., (2002). *Species Sensitivity Distributions in Ecotoxicology*, CRC Press LLC, 1-56670-578-9, Boca Raton, FL.

Potts, G.R. (1986). *The Partridge - Pesticides, Predation and Conservation*. Collins, London, UK.

Powell, G.V.N. (1984). Reproduction by an altricial songbird, the red winged blackbird, in fields treated with the organophosphate insecticide fenthion. *J. Appl. Ecol.* 21:83-95.

Reinecke, S. & Reinecke, A. (2007). The impact of organophosphate pesticides in orchards on earthworms in the Western Cape, South Africa. *Ecotoxicol. Environ. Saf.* 66(2):244-251.

Relyea, Rick A. (2004). Synergistic impacts of malathion and predatory stress on six species of North American tadpoles. *Environ. Toxicol. Chem.* 23(4):1080-1084.

Relyea, R.A. & Hoverman, J.T. (2008). Interactive effects of predators and a pesticide on aquatic communities. *Oikos* 117(11):1647-1658.

Rohr, J.R., Raffel, T.R., Sessions, S.K. & Hudson, P.J. (2008). Understanding the net effects of pesticides on amphibian trematode infections. *Ecol. Appl.* 18(7):1743-1753.

Rose, R. & Dively, G.P. (2007). Effects of insecticide-treated and lepidopteran-active Bt transgenic sweet corn on the abundance and diversity of arthropods *Environ. Entomol.* 36(5):1254-1268.

Sánchez-Bayo, F. & Goka, K. (2006). Ecological effects of the insecticide imidacloprid and a pollutant from antidandruff shampoo in experimental rice fields. *Environ. Toxicol. Chem.* 25(6):1677-1687.

Sánchez-Bayo, F. & Hyne, R.V. (2011). Comparison of environmental risks of pesticides between tropical and nontropical regions. *Integr. Environ. Assess. Manage.* 7(4):577-586

Sánchez-Bayo, F., Yamashita, H., Osaka, R., Yoneda, M. & Goka, K. (2007). Ecological effects of imidacloprid on arthropod communities in and around a vegetable crop. *J. Environ. Sci. Health B* 42(3):279-286.

Schäfer, R.B., Brink, P.J.v.d. & Liess, M. (2011). Impacts of pesticides on freshwater ecosystems. In: *Ecological Impact of Toxic Chemicals*, Sánchez-Bayo, F. et al., editors, pp. 111-137, Bentham Science Publishers, 978-1-60805-121-2, Online

Schäfer, R.B., Caquet, T., Siimes, K., Mueller, R., Lagadic, L. & Liess, M. (2007). Effects of pesticides on community structure and ecosystem functions in agricultural streams of three biogeographical regions in Europe. *Sci. Total Environ.* 382(2-3):272-285.

Schauber, E.M., Edge, W.D. & Wolff, J.O. (1997). Insecticide effects on small mammals: influence of vegetation structure and diet. *Ecol. Appl.* 7(1):143-157.

Schulz, R. & Liess, M. (1999). A field study of the effects of agriculturally derived insecticide input on stream macroinvertebrate dynamics. *Aquat. Toxicol.* 46:155-176.

Sharma, D.C. & Kashyap, N.P. (2002). Impact of pesticidal spray on seasonal availability of natural predators and parasitoids in the tea ecosystem. *J. Biol. Control* 16(1):31-35.

Sheffield, S.R. & Lochmiller, R.L. (2001). Effects of field exposure to diazinon on small mammals inhabiting a semienclosed prairie grassland ecosystem. I. Ecological and reproductive effects. *Environ. Toxicol. Chem.* 20(2):284-296.

Sibly, R.M., Newton, I. & Walker, C.H. (2000). Effects of dieldrin on population growth rates of sparrowhawks 1963–1986. *J. Appl. Ecol.* 37(3):540-546.

Stark, J. & Vargas, R. (2005). Toxicity and hazard assessment of fipronil to *Daphnia pulex* *Ecotoxicol. Environ. Saf.* 62(1):11-16.

Stark, J.D. (2001). Population-level effects of the neem insecticide, Neemix on *Daphnia pulex*. *J. Environ. Sci. Health B* 36(2):457-465.

Story, P. & Cox, M. (2001). Review of the effects of organophosphorus and carbamate insecticides on vertebrates. Are there implications for locust management in Australia? *Wildl. Res.* 28(2):179-193.

Thompson, D.G. (2011). Ecological impacts of major forest-use pesticides. In: *Ecological Impact of Toxic Chemicals*, Sánchez-Bayo, F. et al., editors, pp. 88-110, Bentham Science Publishers, 978-1-60805-121-2, Online

Thompson, H.M. (2001). Assessing the exposure and toxicity of pesticides to bumblebees (*Bombus* sp.). *Apidologie* 32:305-321.

van den Brink, P.J., Wijngaarden, R.P.A.V., Lucassen, W.G.H., Brock, T.C.M. & Leeuwangh, P. (1996). Effects of the insecticide Dursban 4E (active ingredient chlorpyrifos) in outdoor experimental ditches: II. Invertebrate community responses and recovery. *Environ. Toxicol. Chem.* 15(7):1143-1153.

Wadhwa, S. & Gill, R.S. (2007). Effect of Bt-cotton on biodiversity of natural enemies *J. Biol. Control* 21(1):9-15.

Walker, M.K., Stufkens, M.A.W. & Wallace, A.R. (2007). Indirect non-target effects of insecticides on Tasmanian brown lacewing (*Micromus tasmaniae*) from feeding on lettuce aphid (*Nasonovia ribisnigri*). *Biol. Control* 43(1):31-40.

Wang, G., Edge, W.D. & Wolff, J.O. (2001). Rainfall and Guthion 2S interactions affect gray-tailed vole demography. *Ecol. Appl.* 11(3):928-933.

Webber, E.C., Deutsch, W.G., Bayne, D.R. & Seesock, W.C. (1992). Ecosystem-level testing of a synthetic pyrethroid insecticide in aquatic mesocosms. *Environ. Toxicol. Chem.* 11(1):87-105.

Whitford, F. & Showers, W.B. (1987). Impact of insecticides on composition and abundance of ground-dwelling insect fauna in adult European corn borer (Lepidoptera, Pyralidae) action sites in Iowa, USA. *Environ. Entomol.* 16(1):231-236.

Wiktelius, S., Chiverton, P.A., Meguenni, H., Bennaceur, M., Ghezal, F., Umeh, E.-D.N., Egwuatu, R.I., Minja, E., Makusi, R., Tukahirwa, E.et al. (1999). Effects of insecticides on non-target organisms in African agroecosystems: a case for establishing regional testing programmes. *Agric. Ecosyst. Environ.* 75:121-131.

Williams, T., Valle, J. & Viñuela, E. (2003). Is the naturally derived insecticide Spinosad® compatible with insect natural enemies? *Biocontrol Sci. Technol.* 13(5):459-475.

Wirth, E.F., Pennington, P.L., Lawton, J.C., DeLorenzo, M.E., Bearden, D., Shaddrix, B., Sivertsen, S. & Fulton, M.H. (2004). The effects of the contemporary-use insecticide (fipronil) in an estuarine mesocosm. *Environ. Pollut.* 131(3):365-371.

Young, J.E.B., Griffin, M.J., Alford, D.V. & Ogilvy, S.E. (2001). Reducing Agrochemical Use on the Arable Farm: The TALISMAN and SCARAB Projects. London, UK: DEFRA. 416 p.

4

Insecticides as Strategic Weapons for Malaria Vector Control

Mauro Prato[1], Amina Khadjavi[1], Giorgia Mandili[1],
Valerio G. Minero[2] and Giuliana Giribaldi[1]
[1]*Dipartimento di Genetica, Biologia e Biochimica, Facolta' di Medicina e Chirurgia, Universita' di Torino*
[2]*Dipartimento di Medicina e Oncologia Sperimentale, Facolta' di Medicina e Chirurgia, Universita' di Torino*
Italy

1. Introduction

Malaria is a parasitic disease confined mostly to the tropical areas, caused by parasites of the genus *Plasmodium* and transmitted by mosquitoes of the genus *Anopheles*. Annually, nearly a million human deaths, mainly of children ≤5 years of age, are registered among 500 million cases of clinical malaria, whereas 2.37 billion people are estimated to be at risk of infection by *P. falciparum*, the most virulent among *Plasmodia* (Guerra et al., 2008). In 2007, the Bill and Melinda Gates Foundation, rapidly endorsed by the World Health Organization (WHO) and the Roll Back Malaria association, claimed for malaria eradication as the primary goal to be prosecuted (Roberts & Enserink, 2007). In order to achieve such an ambitious objective, several strategies are being adopted, involving multidisciplinary areas such as treatment, chemoprevention, vaccine research, health system assessment and of note vector control (Greenwood, 2008; Khadjavi et al., 2010). Indeed insecticides, which have already been essential components of previous malaria control programs, are supposed to play a key role in the new eradication program, where they will be employed either for indoor spraying or treated bednet approaches (Greenwood, 2008; Khadjavi et al., 2010).

The present chapter will review the status of insecticides currently used for malaria vector control, along with present evidence on their benefits and risks in relation to the available alternatives. After a brief description of the *Plasmodium* life cycle, occurring either in mosquito vector (sexual reproduction) or in human host (asexual replication), the insecticides currently allowed by WHO for malaria vector control, including organophosphates (OP) for larval control and organochlorines (OCs), pyrethroids (PYs) and carbamates (Cs) for the control of adult mosquitoes, will be described; formulation, side effects and cost-effectiveness will be discussed. A special attention will be paid to 1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane (DDT), which is presently used by approximately fourteen countries, while several others are preparing to reintroduce it. Nevertheless, the concerns about the continued use of DDT and the recent reports of high levels of human exposure associated with indoor spraying amid accumulating evidence on chronic health effects will be taken in account. Furthermore, the big issue of growing resistances to the

toxic action of current insecticides, which are spreading almost worldwide, will be focused in a dedicated paragraph. Finally, the existing and future alternatives, either chemical or non-chemical, to the insecticides currently in use will be analyzed focusing on repellents and genetic control. Taken altogether, the data shown in the present chapter could be useful to the reader to better know the present and the future tools available for malaria vector control, in the context of the ongoing malaria eradication program.

Informations on available insecticides, formulations, side effects, resistance, cost-effectiveness, and alternatives have been obtained from literature searches, by using the search engines Scopus and Pubmed. Due to the complexity of the subject, only the most relevant studies were selected, and reviews were prioritized. Old literature was accessed electronically, or hard copies were obtained from libraries. Information on human exposure and health effects is based on reviews published over the past five years and supplemented with recent studies on exposure due to indoor spraying and treated bednets.

2. *Plasmodium* life cycle

Plasmodium species all share the same life cycle, which occurs either in human host (asexual cycle) or in mosquito vector (sexual cycle), as represented in Figure 1.

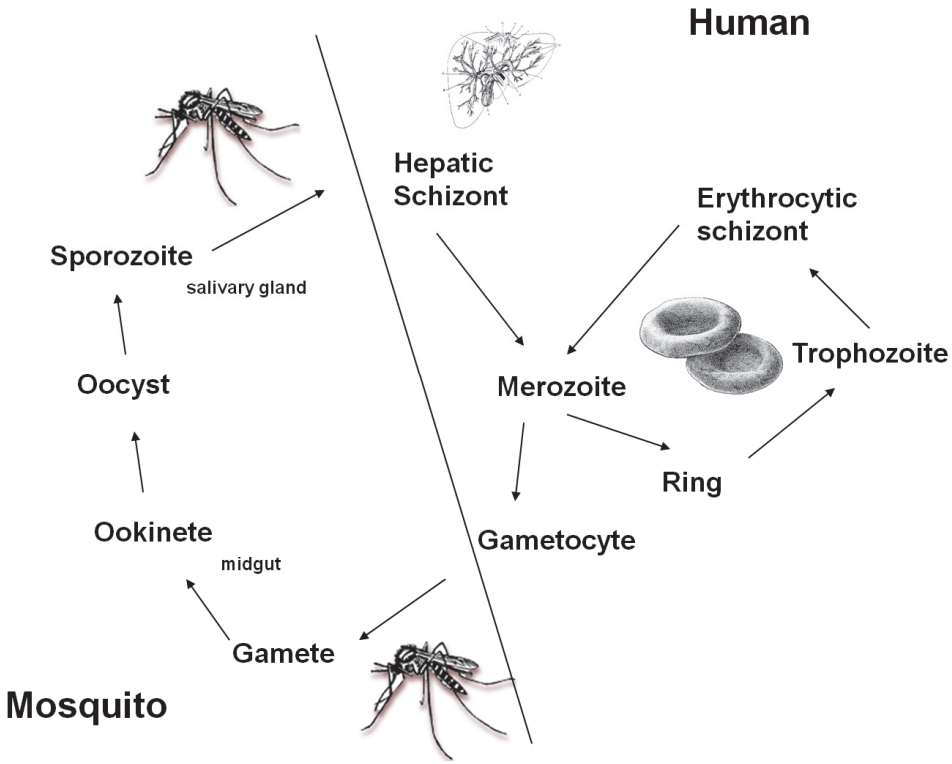


Fig. 1. *Plasmodium* parasite life cycle.

Parasites are transmitted to humans by the females of the *Anopheles* mosquito species. There are about 460 species of *Anopheles* mosquitoes, but only 68 transmit malaria. *Anopheles gambiae*, found in Africa, is one of the major malaria vectors. It is long-living, prefers feeding on humans, and lives in areas near human habitation (Rogier & Hommel, 2011). The malarial infection begins when the sporozoite stage of the parasite, that resides within the salivary gland of the mosquito, halts in the host liver (Menard, 2005). This happens when an infected female bites a healthy person and takes its blood meal, injecting a small amount of saliva into the skin wound. Male mosquito does not feed on blood, hence only female serves as a vector. The saliva contains anti-haemostatic and anti-inflammatory enzymes that disrupt the clotting process and inhibit the pain reaction. Typically, each infected bite contains 5-200 sporozoites which proceed to infect the human vector. Once in the human bloodstream, the sporozoites only circulate for a matter of minutes before infecting liver cells.

2.1 Liver stage in man

After circulating in the bloodstream, sporozoites migrate to the liver and finally infect a hepatocyte, after crossing several Kupffer cells and hepatocytes (Trieu et al., 2006). The sporozoites rapidly grow in size absorbing nourishment to form a large round schizont. The schizont divides by schizogony, a type of asexual reproduction, in which multiple fissions result in the formation of a number of small, spindle-shaped uninucleate cells called merozoites (Rogier & Hommel, 2011). Schizonts rupture and merozoites are released into the sinusoids or venous passages of the liver. This phase of asexual reproduction is called pre-erythrocytic schizogony. The merozoites are immune to medicines and host natural resistance. After a development stage in liver, during which there are no clinical symptoms of disease, merozoites are released into the blood and enter the erythrocytic portion of their life-cycle. A single schizont can produce thousands of merozoites by asexual reproduction.

2.2 Erythrocytic stage in man

The merozoites feed on erythrocytes, become rounded and modify into a trophozoite. During growth, a vacuole appears in the centre of merozoites and the nucleus is pushed to one side; this modification, that is known as “ring stage”, gives it a ring-like appearance. This food vacuole secretes some digestive enzymes, which break down haemoglobin into proteins and haematin. Proteins are used by the parasite as nourishment source, whereas haematin is converted into a waste product called haemozoin, a lipid-enriched ferriprotoporphyrin IX crystal avidly phagocytosed by host immune cells. As a result of phagocytosis, several monocyte functions are impaired, including oxidative burst, bacterial killing, antigen presentation, coordination of erythropoiesis. Moreover, the production of several pro-inflammatory molecules, including cytokines, chemokines and matrix metalloproteinases, as well as the production of anti-apoptotic molecules, such as heat shock protein-27, is enhanced. The overproduction of these host molecules as a response to a parasite product has been proposed to play a crucial role in clinical progress towards complicated malaria, including cerebral malaria, respiratory distress, and placental malaria (Prato et al., 2005, 2008, 2009, 2010a, 2010b, 2010c; Giribaldi et al., 2010; Khadjavi et al., 2010; Prato et al. 2011a, 2011b; Prato 2012; Giribaldi et al., 2011). During their growth, the trophozoites metamorphose into schizonts (Rogier & Hommel, 2011). Schizont appears after a period of about 36 to 40 hours of growth and represents the full-grown trophozoite. The

nucleus of schizont divides in the next 6 to 8 hours to form 12 to 24 daughter nuclei of new merozoite cells in the erythrocyte. This phase of asexual multiplication is known as erythrocytic schizogony. One erythrocytic cycle is completed in 48 hours. Thereafter, the merozoites burst from the red blood cell, and proceed to infect other erythrocytes. The parasite remains in the bloodstream for roughly 60 seconds before entering into another erythrocyte, restarting the process (Cowman & Crabb, 2006). This infection cycle occurs in a highly synchronous fashion, with roughly all of the parasites throughout the blood in the same stage of development. The toxins are liberated into the blood along with the liberation of merozoites. The toxins are then deposed in the liver, spleen and under the skin, so that the host gets a sallow colour. The accumulated toxins cause malaria fever: the patient suffers from chills, shivering, sweating and high temperature. The fever lasts for six to ten hours and then it comes again after every 48 hours with the liberation of a new generation of merozoites. During the erythrocytic stage, some merozoites increase in size to form two types of gametocytes, the macrogametocytes and microgametocytes. The macrogametocytes (female) are large, round with the food laden cytoplasm and a small eccentric nucleus. The microgametocytes (male) are small, with clear cytoplasm and a large central nucleus. This process is called gametocytogenesis. The specific factors and causes underlying this sexual differentiation are largely unknown. The gametocytes take roughly 8–10 days to reach full maturity and do not develop further until they get sucked by the appropriate species of mosquito. If this does not happen, they degenerate and die, because they require lower temperature for further development.

2.3 Life cycle in mosquito

When a female *Anopheles* sucks the blood of a malaria patient, the gametocytes enter along with blood, reaching the stomach and leading to formation of gametes (Aly et al., 2009). Only the gametocytes survive inside the stomach, while the other stages of the parasite, as well as the erythrocytes, are digested. Two types of gametes are formed: the microgametocytes (male) become active and their nucleus divides to produce 6 to 8 haploid daughter nuclei. The nuclei arrange at the periphery. The cytoplasm gives out same number of flagella-like projections. A daughter nucleus enters in each projection. These projections separate from the cytoplasm. This process of formation of microgametes is called exflagellation. From each microgametocyte, 6 to 8 flagella-like active microgametes are formed. The megagametocyte (female) undergoes some reorganization and forms megagametes. Fertilization of the female gamete by the male gamete occurs rapidly after gametogenesis. The fertilization event produces a zygote that remains inactive for some time and then elongates into a worm-like ookinete or vermicule. The zygote and ookinete are the only diploid stages. The ookinete penetrates the wall of the stomach and comes to lie below its outer epithelial layer. It gets enclosed in a cyst formed partly by the zygote and partly by the stomach of mosquito. The encysted zygote is called oocyst. The oocysts absorb nourishment and grow to about five times in size. They protrude from the surface of the stomach as transparent rounded structures. Over a period of 1–3 weeks, the oocyst grows to a size of tens to hundreds of micrometes. During this time, multiple nuclear divisions occur. As a consequence of oocyst maturation, the oocyst divides to form multiple haploid sporozoites. Each oocyst may contain thousands of sporozoites and groups of sporozoites get arranged around the vacuoles. This phase of asexual multiplication is known as sporogony. In the mosquito, the whole sexual cycle is completed in 10 to 21 days. Finally the

oocyst bursts and sporozoites are liberated into the haemolymph of the mosquito. They spread throughout the haemolymph and eventually reach the salivary glands and enter the duct of the hypopharynx. The mosquito now becomes infective and sporozoites get inoculated or injected into the human blood when the mosquito bites, starting a new life cycle. It is estimated that a single infected mosquito may contain as many as 200,000 sporozoites.

3. Insecticides used for malaria vector control

The most prominent classes of insecticides are organochlorines (OCs), organophosphates (OPs), carbamates (Cs), and pyrethroids (PYs). In general, they act by poisoning the nervous system of insects, which is fundamentally similar to that of mammals. A small amount of pesticide can be fatal for an insect, primarily because of its small size and high rate of metabolism. Such an amount is not fatal for humans, but it may still harm. Since the similarities between the nervous system structures make it nearly impossible to design insecticides affecting only insect pests, insecticides may affect non-pest insects, people, wildlife, and pets. Some insecticides harm water quality or affect organisms in other ways; for example, the insecticide carbaryl (a C insecticide, further discussed below) is listed as a carcinogen by the state of California. The newer insecticides are designed to be more specific and less persistent in the environment (Toxipedia, 2011).

3.1 Organochlorines

Chemical structure of OCs is various, but they all contain chlorine, which places them in a larger class of compounds called chlorinated hydrocarbons. These compounds, including DDT, represent a typical example of the potential risks and benefits of insecticide use. OCs have serious unintended consequences, despite the advantage of being cheap and effective against target species. OCs alter and disrupt the movement of ions such as calcium, chloride, sodium, and potassium into and out of nerve cells, but, depending on their specific structure, they may also affect the nervous system in other ways. OCs are very stable, slow to degrade in the environment, soluble in fats (and are therefore readily taken up by insects), and seemingly harmless to mammals; for this reason, at one time, OCs are thought to be ideal. Unfortunately, persistence and fat solubility are very undesirable: OCs can bioaccumulate in the fat of large animals and humans by passing up the food chain. The global use and transport of OCs result in the contamination of wildlife around the globe, including Arctic and Antarctic regions where these insecticides are not used. A decline in the number of birds that prey on animals exposed to DDT is one of the first signs of the unintended consequences. Unexpectedly, DDT causes a thinning of the bird eggshells and results in the death of newborns. OCs like DDT are now largely banned in industrialized countries but they are still manufactured and used in developing countries where they are exposed by the formers.

3.1.1 1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane

DDT is an OC insoluble in water but soluble in most organic solvents, fats, and oils. DDT is not present naturally, but is produced by the reaction of chloral (CCl_3CHO) with chlorobenzene (C_6H_5Cl) in the presence of sulfuric acid, which acts as a catalyst. DDT is a persistent organic pollutant that is extremely hydrophobic and strongly absorbed by soil, where its half life can range from 22 days to 30 years depending on conditions. Routes of

loss and degradation include runoff, volatilization, photolysis and aerobic and anaerobic biodegradation. When applied to aquatic ecosystems DTT is quickly absorbed by organisms and by soil or it evaporates, leaving little amount of DDT dissolved in the water itself (Agency for Toxic Substances and Disease Registry, 2002)

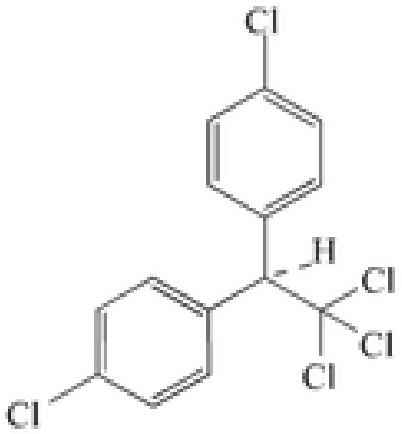


Fig. 2. DDT.

In insects DTT opens sodium ion channels in neurons, causing them to burn spontaneously. This effect leads to spasms and eventual death. For this reason, insects with certain mutations in their sodium channel gene are resistant to DDT and other similar insecticides. DDT resistance is also conferred by up-regulation of genes expressing cytochrome P450 in some insect species (Denholm et al., 2002). In 1955, the WHO commenced a program to eradicate malaria worldwide, relying largely on DDT. The program was initially very successful, eliminating the disease in Taiwan, much of the Caribbean, the Balkans, parts of northern Africa, the northern region of Australia, and a large swath of the South Pacific and dramatically reducing mortality in Sri Lanka and India (Harrison, 1978). However, widespread agricultural use led to resistant insect populations. In many areas, early victories partially or completely reversed, and in some cases rates of transmission even increased (Chapin & Wasserstrom,1981). The program was successful in eliminating malaria only in areas with "high socio-economic status, well-organized healthcare systems, and relatively less intensive or seasonal malaria transmission" (Sadasivaiah et al., 2007). In tropical regions, DDT was less effective due to the continuous life cycle of mosquitoes and poor infrastructure. It was not applied at all in sub-Saharan Africa due to these perceived difficulties.

Through genotoxicity or endocrine disruption DDT may affect human health. DDT may be directly genotoxic, but may also induce enzymes to produce other genotoxic intermediates and DNA adducts [45].

Moreover, based on the results of animal studies, DDT is suspected to cause cancer. By epidemiological studies it is worth demonstrated that DDT causes liver, pancreas and breast cancers. Its contribution in the development of leukemia, lymphoma and testicular cancer is still unclear. Other epidemiological studies suggest that DDT does not cause

multiple myeloma or prostate, endometrium, rectum, lung, bladder, and stomach cancers (Rogan & Chen, 2005; Eskenazi, 2009; Spinelli et al., 2007; McGlynn et al., 2008).

3.2 Organophosphates and Carbamates

OP is the general name for esters of phosphoric acid. These compounds were developed in the 1940s as highly toxic biological warfare agents (nerve gases). Modern derivatives, including sarin and VX, were stockpiled by several countries and now present some difficult disposal problems. In their search for insecticides that would target selected species and would be less toxic to mammals many different OPs have been developed. When the OP Parathion was first used as a replacement for DDT, it was believed to be better and more specific. Unfortunately, Parathion short-term (acute) toxicity is greater than DDT, and this characteristic causes a significant number of human deaths. On the other hand, Cs feature the carbamate ester functional group. Although OPs and Cs have very different chemical structures, they share a similar mechanism of action and will be examined here as one class of insecticides. OPs and Cs affect an important neurotransmitter common to both insects and mammals, the acetylcholine, which is essential for communication of nerve cells. Acetylcholine, released by one nerve cell, initiates communication with another nerve cell, but this stimulation should eventually be stopped. The interruption of this communication is made by removing acetylcholine from the area around the nerve cells. Subsequently, acetylcholine is broken down by a specific enzyme, the acetylcholinesterase. OPs and Cs block the enzyme and disrupt the proper functioning of the nerve cells. Hence, these insecticides are called acetylcholinesterase inhibitors. Structural differences between the various OPs and Cs affect the efficiency and degree of acetylcholinesterase blockage. Nerve gases are highly efficient and permanently block acetylcholinesterase, while the commonly used pesticides block acetylcholinesterase only temporarily. The toxicity of these pesticides presents significant health hazards, and researchers continue to work to develop new insecticides that have fewer unintended consequences.

3.3 Pyrethroids

Synthetic PYs, that were first developed in the 1980s, are one of the newer classes of insecticides; they are loosely based upon the naturally pyrethrum found in *Chrysanthemum* flowers and first commercially used in the 1800s. Their use has increased significantly over the last 20 years. The chemical structure of PYs is quite different from that of OCs, OPs and Cs but the primary site of action is also the nervous system. PYs affect the movement of sodium ions (Na+) into and out of nerve cells that become hypersensitive to neurotransmitters. Structural differences between several PYs can change their toxic effects on specific insects and even mammals. PYs are more persistent in the environment compared to natural pyrethrum, which is unstable in light and breaks down very quickly in sunlight.

3.4 Chemical agents in malaria vector control

The historical successful elimination of malaria in various parts of the world has been achieved mainly by vector control (Harrison, 1978). In addition, the Global Malaria Control Strategy emphasized the need for selective and sustainable preventive measures for reducing malaria transmission (WHO, 1993). In order to control vector-borne diseases, control of mosquitoes is the most important aspect. It is accomplished by application of chemical pesticides against adult-stage mosquitoes. Application of insecticides remains the

primary control tool in the majority of vector control programs throughout the world since early nineteenth century (Breman, 2001). In the twentieth century, after the discovery of DDT, a new era of insect control began (Hassall, 1982). DDT was the first synthetic organic insecticide used for effective vector control with reasonable success. DDT was banned by Environmental Protection Agency in 1972, owing to ecological considerations and opening up a debate between groups for or against the ban. However, the ban exempts its use in public health emergencies like outbreaks of malaria. The restriction permits indoor residual sprays (IRS) of DDT in malaria control until an effective, affordable, and safe alternative is available. In September 2006, based on the increasing scientific evidences, finally, WHO gave a clean bill to use of DDT to fight against malaria in Africa and other areas where the vectors are still susceptible to DDT (WHO, 2006a). However, the debate on the use of DDT is still continuing and will continue until a more effective, affordable, and safe alternative tool is made available.

3.4.1 Indoor residual spraying

Indoor residual spraying (IRS) with insecticides continues to be the mainstay for malaria control and represents an application of stable formulations of insecticides to the interior sprayable surfaces (walls and roofs) of houses to kill the mosquitoes. This affects the malaria transmission by reducing the life span of female mosquitoes thereby reducing density of mosquitoes (WHO, 2006b). Insecticide efficacy depends not only on the molecule intrinsic chemical nature and properties but also on certain technical factors, such as susceptibility of the target vector species to different insecticides, quality of indoor spraying (dose dispensation and coverage), and on residual efficacy. Insecticides recommended by WHO for IRS for control of malaria vectors are given in Table 1.

Insecticide compounds and formulations	Chemical type (2)	Dosage (a.i[a]g/m2)	Mode of action	Duration of effective action (months)
DDT WP	OC	1–2	Contact	>6
Malathion WP	OP	2	Contact	2–3
Fenitrothion WP	OP	2	Contact & airborne	3–6
Pirimiphos-methyl WP, EC	OP	1–2	Contact & airborne	2–3
Bendiocarb WP	C	0.1–0.4	Contact & airborne	2–6
Propoxur WP	C	1–2	Contact & airborne	3–6
Alpha-cypermethrin WP, SC	PY	0.02–0.03	Contact	4–6
Bifenthrin	PY	0.025–0.05	Contact	3–6
Cyfluthrin WP	PY	0.02–0.05	Contact	3–6
Deltamethrin WP, WG	PY	0.02–0.025	Contact	3–6
Etofenprox WP	PY	0.1–0.3	Contact	3–6
Lambda-cyhalothrin WP, CS	PY	0.02–0.03	Contact	3–6

Formulations: *CS* capsule suspension; *EC* emulsifiable concentrate; *WP* wettable powder; *OC* Organochlorines; *OP* Organophosphates; *C* Carbamates; *PY* Pyrethroids; [a] *a.i.* active ingredient

Table 1. Insecticides recommended for IRS against malaria vectors.

3.4.2 Space spraying

Space spraying/fogging, which is produced by rapidly heating the liquid chemical to form very fine droplets that resemble smoke or fog, is the process of application of a pesticide . It is primarily reserved for application during emergency situations for halting epidemics or rapidly reducing adult mosquito populations resulting in decrease of transmission (CDC, 2009). It is effective as a contact poison with no residual effect. Space spraying must coincide with the peak activity of adult mosquitoes, because resting mosquitoes are often found in areas that are out of reach to the applied insecticides (e.g., under leaves, in small crevices). The best moment to kill adult mosquitoes by fogging is at dusk, when they are most active in forming swarms. The most commonly used products are natural pyrethrum extract, synthetic PYs, and Malathion. WHO recommended insecticides for space sprays are listed in Table 2.

Insecticide	Chemical type	Dosage of a.i[a] (g/ha)	
		Cold aerosol	Thermal fog
Boiresmethrin	PY	5	10
Cyfluthrin	PY	1–2	1–2
Cypermethrin	PY	1–3	–
Cyphenothrin	PY	2–5	5–10
Deltamethrin	PY	0.5–1.0	–
D-phenothrin	PY	5–20	–
Etofenprox	PY	10–20	10–20
Fentirothion	OP	250–300	250–300
Malathion	OP	112–600	500–600
Permethrin	PY	5	10
Pirimphos-methyl	OP	230–330	180–200
Resmethrin	PY	2–4	4
d,d-trans-cyphenothrin	PY	1–2	2.5–5

[a] *a. i.* active ingredient

Table 2. Insecticides suitable for application as cold aerosol ULV sprays or thermal fogs for mosquito control.

3.4.3 Insecticide-treated nets

Mosquito nets effectively prevent malaria transmission by forming a physical barrier between insects and man. Insecticide-treated nets (ITNs), impregnated with PYs, were introduced in the place of untreated nets, that are not a perfect barrier, not only in order to decrease the man–mosquito contact by deterrence or excito-irritability but also to kill the mosquito with its residual insecticidal activity. They are more effective than untreated nets with >70% protection and are proved to be a cost-effective prevention method against malaria (D'Alessandro et al., 1995). WHO-recommended insecticide products for the treatment of mosquito nets for malaria vector control are given in Table 3.

1. Conventional Treatment		
Insecticide	**Formulation**	**Dosage (mg/m^{2} net)**
Alpha-cypermethrin	Suspension concentrate 10%	20–40
Cyfluthrin	Emulsion, oil in water 5%	50
Deltamethrin	Suspension concentrate 1%; Water dispersible tablet 25% and WT 25% + binder [3]	15–25
Etofenprox	Emulsion, oil in water 10%	200
Lambda-cyhalothrin	Capsule suspension 2.5%	10–15
Permethrin	Emulsifiable concentrate 10%	200–500
2. Long-lasting treatment		
Product name	**Product type**	**Status of WHO recommendation**
ICON® MAXX	Lambda-cyhalothrin 10% CS + binder Target dose of 50 mg/m2	Interim

Table 3. WHO-recommended insecticide products for the treatment of mosquito nets for malaria vector control.

3.4.4 Long-lasting insecticidal materials

The rapid loss of efficacy of ITNs due to washing and to the associated low-retreatment rates of the nets limits the operational effectiveness of an ITN program (Lines, 1996). Long-lasting insecticidal nets (LLINs) reduce human–mosquito contact, which results in lower sporozoite and parasite rates. The biological activity generally lasts as long as the net itself (3–4 years for polyester nets and 4–5 years for polyethylene nets) (WHO, 2005). A list of WHO-recommended long-lasting insecticidal mosquito nets for use in public health is given in Table 4. Only five brands of LLINs are currently recommended by the WHO Pesticide Evaluation Scheme, and Olyset® net is the only one which currently granted full recommendation (N'Guessan et al., 2001; Teklehaimanot et al., 2007), while Perma-Net-2.0®, Duranet-®, Net Protect-®, and Interceptor-®, including long-lasting insecticide treatment kits K-OTab1-2-3® and ICON-MAXX® (Sinden, 2007), are approved as an interim recommendation.

Also treatments of screens, curtains, canvas tents, plastic sheet, tarpaulin, etc., with insecticides may provide a cheap and practical solution for malaria vector control. Effectiveness of treated screen and curtains can be comparable to that of mosquito nets. Different types of long-lasting insecticide impregnated materials are under field trials in different countries. The residual insecticides in insecticide-treated wall lining (ITWL) are durable and maintain control of insects significantly longer than IRS and may provide an effective alternative or additional vector control tool to ITNs and IRS (Munga et al., 2009).

4. Insecticide resistance

A major concern on the use of currently available insecticides for malaria control is represented by increasing insecticide resistance (Enayati & Hemingway, 2010). For example, DDT was first introduced for mosquito control in 1946; however, already in 1947 the first cases of DDT resistance occurred, and up to now DDT resistance at various levels

Product name	Product type	Status of WHO recommendation
DawaPlus® 2.0	Deltamethrin coated on polyester	Interim
Duranet ®	Alpha-cypermethrin incorporated into polyethylene	Interim
Interceptor ®	Alpha-cypermethrin coated on polyester	Interim
Netprotect®	Deltamethrin incororated into polyethylene	Interim
Olyset®	Permethrin incorporated into polyethylene	Full
PermaNet ®2.0	Deltamethrin coated on polyester	Full
PermaNet® 2.5	Deltamethrin coated on polyester with strengthened border	Interim
PermaNet® 3.0	Combination of deltamethrin coated on polyester with strengthened border (side panels) and deltamethrin and PBO incorporated into polyethylene (roof)	Interim

Table 4. WHO-recommended long-lasting insecticidal mosquito nets for use in public health.

has been reported for > 50 species of *Anopheles* mosquitoes, including many vectors of malaria (Hemingway & Ranson, 2000). Unfortunately, the introduction of new other insecticides for malaria control, including OPs, Cs, and PYs, improved malaria control strategy only partially, since resistance has tended to follow the switches in insecticides (Hemingway & Ranson, 2000).

In the past, the use of DDT in agriculture was considered a major cause of its resistance in malaria vectors, as many vectors breed in agricultural environments (Mouchet, 1988). At present, DDT resistance is thought to be triggered further by the use of synthetic PYs (Diabate et al., 2002). Indeed, DDT and PYs share a common target, thus facilitating the development of a cross-resistance mechanism (Martinez-Torres et al., 1998). In addition, evidence of increased frequency of resistance genes due to IRS or ITN programs is quite alarming (Karunaratne & Hemingway 2001; Stump et al., 2004): PYs, the only class approved for use on ITNs (Zaim M et al 2000), are being increasingly deployed in IRS programmes in Africa and there has been a dramatic increase in reports of PY resistance in malaria vectors over the past decade (Santolamazza et al., 2008); moreover, PYs are also widely used in the control of agricultural pests worldwide (Ranson et al., 2011).

Typically, two major mechanisms are assumed to be responsible for insecticide resistance: a) changes in the insecticide target site (mutations in the sodium channel, acetylcholinesterase and GABA receptor genes) that reduce its binding; b) increased rates of insecticide metabolism (alterations in the levels or activities of detoxification proteins) and reduced insecticide ability to reach the target site (Hemingway et al., 2004; Ranson et al., 2011).

These mechanisms, alone or in combination, lead to resistance, sometimes at an extremely high level, to all of the available classes of insecticides (Hemingway et al., 2004).

4.1 Target site resistance

As previously discussed, OPs, Cs, OCs, and PYs all target the nervous system (Enayati & Hemingway, 2010). Single base point mutations are the most common cause of target-site resistance, changing the properties of these target sites, and reducing their susceptibility to insecticide binding (Hemingway & Ranson, 2000; Enayati & Hemingway, 2010).

4.1.1 Voltage-gated sodium channel

PYs and OCs target the voltage-gated sodium channel in insect neurons (Davies, T.G. et al. 2007). Insecticide binding delays closure of the sodium channel prolonging action potential and causing repetitive neuron firing, paralysis and eventual death of the insect (Ranson, 2011). Mutations in the sodium channel conferred by DDT and PY resistance are known as knockdown resistance (kdr), so-called because insects with these alleles can withstand prolonged exposure to insecticides without being 'knocked-down' (Hemingway et al., 2004; Hemingway & Ranson, 2000; Ranson, 2011). The kdr is due to changes in the affinity between the insecticide and its binding site on the sodium channel, as a consequence of single or multiple substitutions in the sodium channel gene (Martinez-Torres et al., 1998). 1014 residual aminoacid replacement, which consists in substitution of the leucine residue with an alternative phenylalanine or serine, does not appear to interact directly with the insecticide but is predicted to alter channel activation kinetics (O'Reilly A.O. et al. 2006,Enayati A. and Hemingway J. 2010; Ranson H. et al 2011). However, even though the association between kdr and resistance to PYs and DDT is clear, it is not well understood whether this allele resistance alone is sufficient to lead to control failure (Ranson et al., 2011).

4.1.2 Acetylcholinesterase

The molecular target of OPs and Cs is acetylcholinesterase (AChE) (Enayati & Hemingway, 2010). AChE has a key role in the nervous system, terminating nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine on the post-synaptic nerve membrane (Hemingway & Ranson, 2000; Hemingway et al., 2004). The insecticides inhibit enzyme activity by covalently phosphorylating or carbamylating the serine residue within the active site (Corbett, 1984). Mutations in AChE gene in OP- and C-resistant insects result in a decreased sensitivity to inhibition of the enzyme by these insecticides (Hemingway & Ranson, 2000).

4.1.3 GABA receptor

The target site of cyclodiene insecticides, such as dieldrin, and of fipronil, a phenyl pyrazole insecticide, is the type A receptor for the neurotransmitter γ-aminobutyric acid (GABA). The GABA receptor is a widespread inhibitory neurotransmission channel in the central nervous system and neuromuscular junctions of insects (Hemingway & Ranson, 2000). GABA receptor binding elicits rapid gating of an integral chloride selective ion channel. Mutations at a single codon in the Rdl (resistance to dieldrin) gene (encoding one receptor subunit), from an alanine residue to a serine or more rarely to a glycine, have been documented in all dieldrin- resistant insect species to date (ffrench-Constant et al., 1998). This mutation appears to confer both insensitivity to the insecticide and a decreased rate of desensitization (Hemingway et al., 2004).

4.2 Metabolic resistance

Metabolic resistance occurs when elevated activity of one or more enzymes results in a sufficient sequester or detoxification of the insecticide before it reaches the target site (Ranson et al., 2011). Increased expression of the genes encoding the major xenobiotic metabolizing enzymes is the most common cause of insecticide resistance in mosquitoes (Hemingway & Ranson, 2000).

Three major enzyme groups are responsible for metabolically based resistance to OCs, OPs, Cs, and PYs: a) glutathione S-transferase (GST), like DDT-dehydrochlorinase, which was

first recognized as a GST in the house fly, *Musca domestica*; b) esterases, often involved in OP, C, and to a lesser extent, PY resistance; and c) monooxygenases, involved in PY metabolism, OP activation and/or detoxication and, to a lesser extent, C resistance (Hemingway & Ranson, 2000).

4.2.1 Glutathione S-transferases

Several studies have shown that insecticide-resistant insects have elevated levels of GST activity, which has been implicated in resistance to at least four classes of insecticides. GSTs are dimeric multifunctional enzymes that play a role in detoxification of a large range of xenobiotics through catalysis of the nucleophilic attack of reduced glutathione on the electrophilic centers of lipophilic compounds. For mosquitoes multiple forms of these enzymes have been reported (Hemingway & Ranson, 2000). Higher enzyme activity is usually due to an increased amount of one or more GST enzymes, either as a result of gene amplification or more commonly through increases in transcriptional rate, rather than qualitative changes in individual enzymes (Ranson & Hemingway, 2004).

The DDT dehydrochlorinase reaction proceeds via a base abstraction of hydrogen, catalyzed by the thiolate anion generated in the active site of the GST, leading to the elimination of chlorine from DDT and generating DDE (Prapanthadara et al., 1995). These GSTs also act as a secondary detoxification route for OPs, resulting in cross-resistance to insecticides such as fenitrothion.

Detoxification of OPs occurs via an O-dealkylation or O-dearylation reaction. In O-dealkylation, glutathione is conjugated with the alkyl portion of the insecticide (Oppenoorth et al., 1979), whereas the reaction of glutathione with the leaving group (Chiang & Sun, 1993) is an O-dearylation reaction. GSTs can also catalyse the secondary metabolism of OP insecticides (Hemingway et al., 2004).

GSTs have no direct role in the metabolism of PY insecticides but they play a very important role in conferring resistance to this insecticide class by reducing oxidative damage and detoxifying the lipid peroxidation products induced by PYs (Vontas et al., 2001). GSTs may also protect against PY toxicity in insects by sequestering the insecticide (Kostaropoulos et al., 2001).

4.2.2 Esterases

Over-production of non-specific carboxylesterases as response to OP and C insecticide selection pressure has been documented in numerous arthropod species including mosquitoes (Hemingway & Karunaratne, 1998). In OP-susceptible insects, the active oxon analogues of the insecticides act as esterase inhibitors, because they are poor substrates with a high affinity for the enzymes. Esterases from resistant insects are more reactive with insecticides than their counterparts from susceptible insects and so they sequester the oxon analogues protecting the acetylcholinesterase target site (Karunaratne et al., 1995). The predominant cause of this excessive enzyme synthesis is amplification of the genes (Mouches et al., 1986; Vaughan & Hemingway, 1995; Vaughan et al., 1995), although up-regulated transcription without an underlying gene amplification event has been reported (Rooker et al., 1996). In some resistant mosquito species, elevated carboxylesterase activity involves rapid hydrolysis of the insecticide, rather than increased sequestration (Hemingway et al., 2004). This mechanism is almost always found in association with Malathion resistance, and gives a much narrower cross-resistance

spectrum (in some cases Malathion-specific) than the amplified esterase-based mechanism. Although the genetic alterations generating these qualitative changes have not yet been identified in mosquito populations, several data obtained from other arthropods suggest that only one or two amino acid mutations may be responsible (Hemingway et al., 2004).

4.2.3 Monooxygenases

Monooxygenases are involved in the metabolism of PYs and in the activation and/or detoxification of OP insecticides (Hemingway & Ranson, 2000). The monooxygenases are a complex family of enzymes found in most organisms, including insects, involved in the metabolism of xenobiotics. The P450 monooxygenases are generally the rate-limiting enzyme step in the chain. Cytochrome P450-dependent monooxygenases are an important and diverse family of hydrophobic, haem-containing enzymes involved in the metabolism of numerous endogenous and exogenous compounds and of virtually all insecticides. It lead to activation of the molecule in the case of OP insecticides, or more generally to detoxification. P450 enzymes bind molecular oxygen and receive electrons from NADPH to introduce an oxygen molecule into the substrate (Hemingway & Ranson, 2000). There are many reports demonstrating elevated P450 monooxygenase activities in insecticide-resistant mosquitoes, frequently in conjunction with altered activities of other enzymes (Hemingway et al., 2004).

4.3 Cuticular resistance

Some mosquitoes have also evolved thicker or altered cuticles, reducing penetration of the insecticide (Stone & Brown, 1969; Apperson & Georghiou, 1975). Obviously this is not the main resistance mechanism used by pests, since the major route of insecticide delivery is by ingestion. However, in malaria control, insecticides are typically delivered on bed nets or on wall surfaces, and uptake of insecticides is primarily through the appendages. Hence an increase in the thickness of the tarsal cuticle, or a reduction in its permeability to lipophilic insecticides, could have a major impact on the bioavailability of insecticide *in vivo* (Ranson et al., 2011).

4.4 Behavioural resistance

Mosquitoes are able to change their behaviour as a result of intensive indoor use of insecticides, but there are currently insufficient data to assess whether these behavioural avoidance traits are symptomatic of genetic or adaptive responses (Bogh et al., 1998).

Several insecticides such as DDT and permethrin influence behavioural changes in the insect by reducing the rate of mosquito entry into houses, by increasing the rate of early exit from houses and by inducing a shift in biting times (Lines et al., 1987; Mbogo et al., 1996; Mathenge et al., 2001). Mosquitoes may also express a change in host preference because their favoured hosts under the ITN can not be reached (Takken, 2002).

In vector control-free areas, mosquitoes are mostly collected in bedrooms. The excito-repellent effect of PYs forces mosquitoes to leave rooms to outdoors, thus explaining the reduction of indoor biting (Takken, 2002). There is a clear need for robust controlled studies to quantify the extent of this behavioural change, and to assess whether scale-up of ITNs and/or IRS could increase importance of outdoor transmission of malaria and new tools against outdoors malaria vectors might be required (Ranson et al., 2011).

5. Future perspectives and possible alternatives to insecticides

Regular monitoring for insecticide resistance is essential in order to react promptly to prevent vector control compromission. Once resistance reaches very high levels, strategies to restore susceptibility are unlikely to be effective (Ranson et al., 2011).
Effective monitoring and decision support systems can be used to detect insecticide resistance at an early stage, which should lead to the implementation of changes in insecticide policy (Sharp et al., 2007). However, the practice of using an insecticide until resistance becomes a limiting factor is rapidly eroding the number of suitable insecticides for vector control (Hemingway & Ranson, 2000) and the choice of unrelated insecticides remains limited (Nauen, 2007).
Rotations, mosaics, and mixtures have all been proposed as resistance management tools (Hemingway & Ranson, 2000): they could delay the development and/or spread of resistance (Curtis C.F. et al., 1998), but cannot prevent it (Penilla et al., 2006).
Efforts are being made to expand the number of available insecticide classes. One initiative is the Innovative Vector Control Consortium (IVCC), a Product Development Partnership, established in 2005 to stimulate the search for alternative active ingredients or improved formulations of insecticides for vector control, and several promising leads are now being evaluated in laboratory and field trials (Ranson et al., 2011; Enayati & Hemingway, 2010). With this goal, also the discovery of new potential targets can be important. For example the sequencing of the *Anopheles gambiae* genome has also been exploited by several groups to identify the range and function of olfactory receptors in the mosquito, with the aim of developing new attractants and repellents (Enayati & Hemingway, 2010).

5.1 Chemical alternatives: repellents

In order to push away mosquitoes, which usually are attracted by the moisture, warmth, carbon dioxide or estrogens from human skin, a large spectrum of repellents have been developed and are currently used; these substances, manufactured in several forms, including aerosols, creams, lotions, suntan oils, grease sticks and cloth-impregnating laundry emulsions, are usually applied on the skin or clothes, and produce a vapor layer characterized by bad smell or taste to insects (Brown & Hebert, 1997). The ideal repellent should satisfy several criteria: a) have long-lasting effectiveness; b) do not irritate human skin; c) have a bad odor only to mosquitoes but not to people; d) have no effects on clothes; e) be inert to plastics commonly used, such as glasses or bracelets; f) be chemically stable; and g) be economical (Brown & Hebert, 1997).
The list of main insect repellents, some of which are also used as insecticides, includes N,N-diethyl-3-methylbenzamide (DEET), permethrin, picaridin, indalone, and botanicals.
DEET has been considered the most broad-spectrum and efficacious repellent for sixty years, and is currently used on the skin or clothes. Its mechanism of action is to provide a vapor barrier with a bad odor capable to push down mosquitoes. Among side effects, central nervous system, cardiovascular, cutaneous symptoms have been reported, but generally they were related to overuse o incorrect use of the product (Osimitz & Grothaus, 1995).
Permethrin is a synthetic PY with also repellent properties. Its mechanism of action requires direct contact with the insect; thus it is not recommended for skin application. It is commonly used in agriculture, and can be used on clothing, shoes, bed nets and camping

gear. High doses might induce neurotoxic effects, eye and skin irritation, reproductive anomalies, and immune system alterations (Cox, 1998).
Picaridin (2-(2-hydroxyethil)-1-piperidinecarboxylic acid 1-methylpropyl ester) has been used for almost a decade in Australia, and therefore extended to Europe and America. Like DEET, it produces a repellent vapor barrier. Interestingly, no side effects have been reported, and in the future it might be useful in areas endemic for malaria; unfortunately, at present it is not recommended for children younger than 2 years, the most susceptible target of *Plasmodium* in tropical areas (Solberg et al., 1995).
Indalone (butyl 3,4-dihydro-2,2-dimethyl-4-oxo-2H-pyran-6-carboxylate) is a contact or gustatory repellent, slightly volatile, and contact with the treated surface is required to push away the insects (Brown & Hebert, 1997)
Botanicals contain one of several essential plant oils including oil of lemon eucalyptus, soybean oil, geraniol or oil of citronella. Natural products might be safer for human use than synthetic compounds (Katz et al., 2008). Among natural insect repellents, the most commonly used is oil of citronella, an essential oil extracted from the long narrow leaves of a perennial grass from tropical Asia. However, despite its repellent properties, citronella seems not to be useful for malaria vector control; indeed, it is commercially available only as Natrapel (10% citronella), which unfortunately is not effective against mosquitoes, and as Green Ban (a mix of citronella, peppermint, cajaput grass, myrrh and sassafras), which is the most expensive insect repellent on the market (Brown & Hebert, 1997). Nevertheless, natural plants clearly represent a large, promising and almost yet unexplored area for research of new repellent molecules useful also to malaria community.

5.2 Non-chemical alternatives: genetic control

The development of non-chemical strategies alternative to insecticides and repellents is presently on study. Genetic control appears a promising tool, comprising all methods by which a mechanism for pest or vector control is introduced into a wild population through mating. These include the sterile insect release method or the sterile insect technique (SIT), through which males are sterilized by irradiation or other means and released to mate with wild females, leading them to lay sterile eggs. Additionally, the introduction of genetic factors into wild populations aimed to make pests harmless to humans might be relevant (Pates & Curtis, 2005). Finally novel approaches against vector borne diseases include transgenesis and paratransgenesis to reduce vector competence (Coutinho-Abreu et al., 2010).
For vector transgenesis, the goal is to transform vectors with a gene (or genes) whose protein(s) impair pathogen development. Several mosquito species vectors of different parasites and viruses have been transformed. Some of the transformed mosquitoes were shown capable of blocking pathogen development via tissue-specific expression of molecules impairing the pathogen attachment to the midgut (Ito et al., 2002), or activating some biochemical pathways detrimental to pathogen survival (Franz et al., 2006). Paratransgenesis aims to reduce vector competence by genetically manipulating symbionts. Transformed symbionts are spread maternally or via coprophagy across an insect population (Durvasula et al., 1997). Unfortunately, although these approaches are potentially promising, they remain a complex approach with a limited use (Coutinho-Abreu et al., 2010).

6. Conclusion

The goal to globally eradicate malaria worldwide, established in 2007 by the Bill and Melinda Gates Foundation and rapidly endorsed by the World Health Organization (WHO) and the Roll Back Malaria association, is certainly ambitious. The combination of parallel vector control approaches, either based on current knowledge of benefits and risks of available insecticides or on future research on new promising tools, including chemical agents like repellents or non-chemical strategies such as genetic control, might be helpful in order to reach such an objective. Therefore, it represents an intriguing but hopefully affordable challenge for all the malaria research community.

7. Acknowledgment

The publication charges of this chapter were sustained by kind private donation from Mrs. Franca Squazza. MP holds a professorship granted by Università degli Studi di Torino and Azienda Sanitaria Locale-19 (ASL-19). The authors have no conflicting financial interests.

8. References

Agency for Toxic Substances and Disease Registry. (September 2002). *Toxicological Profile: for DDT, DDE, and DDE,* U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES Public Health Service, Retrieved from http://www.atsdr.cdc.gov/toxprofiles/tp35.pdf

Aly, A.S.; Vaughan, A.M. & Kappe, S.H. (2009). Malaria parasite development in the mosquito and infection of the mammalian host. *Annual Review of Microbiology*, Vol.63, (October 2009), pp. 195-221, ISSN 0066-4227

Apperson, C.S. & Georghiou, G.P. (1975). Mechanisms of resistance to organophosphorus insecticides in Culex tarsalis. *Journal of Economic Entomology*, Vol.68, No.2, (April 1975), pp. 63–78, ISSN 0022-0493

Bøgh, C.; Pedersen, E.M.; Mukoko, D.A. & Ouma, J.H. (1998). Permethrin-impregnated bednet effects on resting and feeding behaviour of lymphatic filariasis vector mosquitoes in Kenya. *Medical and Veterinary Entomology,* Vol.12, No.1, (January 1998), pp. 52–59, ISSN 1365-2915

Breman, J.G. (2001). The ears of the hippopotamus: manifestations, determinants and estimates of the malaria burden. *American Journal of Tropical Medicine and Hygiene*, Vol.64, No.1-2Suppl, (January-February 2001), pp. 1–11, ISSN 0002-9637

Brown, M. & Herbert, A.A. (1997). Insect repellents: An overview. *Journal of the American Academy of Dermatology*, Vol.36, No.2Pt1, (February 1997), pp. 243-249, ISSN 1097-6787

CDC. (2009). Malaria - Vector Control. In: *Center for disease control and prevention*, 21 April 2009, Available from http://www.cdc.gov/malaria/ control_prevention/vector_control.htm.

Chapin, G. & Wasserstrom, R. (1981). Agricultural production and malaria resurgence in Central America and India. *Nature*, Vol.293, No.5829, (September 1981), pp. 181-185, ISSN 0028-0836

Chiang, F. & Sun, C. (1993). Glutathione transferase isozymes of diamondback moth larvae and their role in the degradation of some organophosphorus insecticides. *Pesticide Biochemistry and Physiology*, Vol.45, No.1, (January 1993), pp. 7–14, ISSN 0048-3575

Cohn, B.A.; Wolff, M.S.; Cirillo, P.M. & Sholtz, R.I. (2007). DDT and breast cancer in young women: new data on the significance of age at exposure. *Environmental Health Perspectives,* Vo.115, No.10, (July 2007), pp. 1406–14, ISSN 1552-9924

Corbett, J.R.; Wright, K. & Baillie, A.C. (1984). *The Biochemical Mode of Action of Pesticides*. (2nd edition), Academic Press Inc, ISBN 0-12187-850-3, London, U.K.

Coutinho-Abreu, I.V.; Zhu, K.Y. & Ramalho-Ortigao, M. (2010). Transgenesis and paratransgenesis to control insect-borne diseases: Current status and future challenges. *Parasitology International*, Vol.59, No1, (March 2010), pp. 1–8, ISSN 1873-0329

Cowman, A.F. & Crabb, B.S. (2006). Invasion of Red Blood Cells by Malaria Parasites. *Cell*, Vol.124, No.4, (February 2006), pp. 755–766, ISSN 0092-8674

Cox, C. (1998). Permethrin. *Journal of Pesticide Reform*, Vol.18, No.2, (1998), pp.14-20, ISSN 0893-357X

Curtis, C.F.; Miller, J.E.; Hodjati, M.H.; Kolaczinski, J.H. & Kasumba, I. (1998). Can anything be done to maintain the effectiveness of pyrethroid-impregnated bednets against malaria vectors? *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, Vol.353, No.1376, (October 1998), pp. 1769-75, ISSN 1471-2970

D'Alessandro, U.; Olaleye, B.O.; McGuire, W.; Thomson, M.C.; Langerock, P.; Bennett, S. & Greenwood, B.M. (1995). A comparison of the efficacy of insecticide-treated and untreated bed nets in preventing malaria in Gambian children. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, Vol.89, No.6, (November-December 1995), pp. 596–598, ISSN 1878-3503

Davies, T.G.; Field, L.M.; Usherwood, P.N. & Williamson, M.S. (2007). DDT, pyrethrins, pyrethroids and insect sodium channels. *IUBMB Life*, Vol.59, No.3, (March 2007), pp. 151–162, ISSN 1521-6551

Denholm, I.; Devine, G.J. & Williamson, M.S. (2002). Evolutionary genetics. Insecticide resistance on the move. *Science*, Vol.297, No.5590, (September 2002), pp. 2222–2223, ISSN 1095-9203

Diabate, A.; Baldet, T.; Chandre, F.; Akoobeto, M.; Guiguemde, T.R.; Darriet, F.; Brengues, C.; Guillet, P.; Hemingway, J.; Small, G.J. & Hougard, J.M. (2002). The role of agricultural use of insecticides in resistance to pyrethroids in Anopheles gambiae s.l. in Burkina Faso. *American Journal of Tropical Medicine and Hygiene*, Vol.67, No.6, (December 2002), pp. 617–622, ISSN 0002-9637

Durvasula, R.V.; Gumbs, A.; Panackal, A.; Kruglov, O.; Aksoy, S.; Merrifield, R.B.; Richards, F.F. & Beard, C.B. (1997). Prevention of insect-borne disease: an approach using transgenic symbiotic bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.94, No.7, (April 1997), pp. 3274–3278, ISSN 1091-6490

Enayati, A. & Hemingway, J. (2010). Malaria Management: Past, Present, and Future. *Annual Review of Entomology*, Vol.55, (January 2010), pp. 569–591, ISSN 1545-4487

Eskenazi, B. ; Chevrier, J.; Goldman, L.R.; Anderson, H.A.; Bornman, M.S.; Bouwman, H.; Chen, A.; Cohn, B.A.; de Jager, C.; Henshel, D.S.; Leipzig, F.; Leipzig, J.S.; Lorenz, E.C.; Snedeker, S.M. & Stapleton, D. (2009). The Pine River Statement: Human

Health Consequences of DDT Use. *Environmental Health Perspectives*, Vol.117, No.9, (September 2009), pp. 1359-1367, ISSN 1552-9924

ffrench-Constant, R.H.; Pittendrigh, B.; Vaughan, A. & Anthony, N. (1998). Why are there so few resistance-associated mutations in insecticide target genes? *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, Vol.353, Vol.1376, (October 1998), pp. 1685–1693, ISSN 1471-2970

Franz, A.W.; Sanchez-Vargas, I.; Adelman, Z.N.; Blair, C.D.; Beaty, B.J.; James, A.A. & Olson, K.E. (2006). Engineering RNA interference-based resistance to dengue virus type 2 in genetically modified Aedes aegypti. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.103, No11, (March 2006), pp. 4198–203, ISSN 1091-6490

Giribaldi, G.; Prato, M.; Ulliers, D.; Gallo, V.; Schwarzer, E.; Akide-Ndunge, O. B.; Valente, E.; Saviozzi, S.; Calogero, R.A. & Arese, P. (2010). Involvement of inflammatory chemokines in survival of human monocytes fed with malarial pigment. *Infection and Immunity*, Vol.78, No.11, (August 2010), pp. 4912-4921, ISSN 1098-5522

Giribaldi, G.; Valente, E.; Khadjavi, A.; Polimeni, M. & Prato, M. (2011). Macrophage Inflammatory Protein-1alpha mediates Matrix Metalloproteinase-9 enhancement in human adherent monocytes fed with malarial pigment. *Asian Pacific Journal of Tropical Medicine*, in press, (2011), ISSN 1995-7645

Greenwood, BM. (2008). Control to elimination: implications for malaria research. *Trends in Parasitology*, Vol.24, No.10, (October 2008), pp. 449-454, ISSN 1471-5007

Guerra, C.A.; Gikandi, P.W.; Tatem, A.J.; Noor, A.M.; Smith, D.L.; Hay, S.I. & Snow, R.W. (2008). The limits and intensity of Plasmodium falciparum transmissionn: implications for malaria control and elimination worldwide. *PLoS Medicine*, Vol.5, No.2, (February 2008) pp. 300-311, ISSN 1549-1676

Harrison, G. (1978). *Mosquitoes, Malaria, and Man: A History of the Hostilities Since 1880*, John Murray, ISBN 0-71953-780-8, London, UK.

Hassall, K.A. (1982). *The chemistry of pesticides: Their metabolism, mode of action and uses in crop protection*, The MacMillan Press Ltd, ISBN 3-52725-969-4, London, U.K.

Hemingway, J. & Karunaratne, S.H.P.P. (1998). Mosquito carboxylesterases: a review of the molecular biology and biochemistry of a major insecticide resistance mechanism. *Medical and Veterinary Entomology*, Vol.12, No.1, (January 1998), pp. 1–12, ISSN 1365-2915

Hemingway, J. & Ranson, H. (2000). Insecticide resistance in insect vectors of human disease. *Annual Review of Entomology*, Vol.45, (2000), pp. 371–391, ISSN 1545-4487

Hemingway, J.; Hawkes, N.J.; McCarroll, L. & Ranson, H. (2004). The molecular basis of insecticide resistance in mosquitoes. *Insect biochemistry and molecular biology*, Vol.34, No.7, (July 2004), pp. 653–65, ISSN 1879-0240

Ito, J.; Ghosh, A.; Moreira, L.A.; Wimmer, E.A. & Jacobs-Lorena, M. (2002). Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite. *Nature*, Vol.417, No.6887, (May 2002), pp. 452–455, ISSN 0028-0836

Karunaratne, S.H.P.P.; Hemingway, J.; Jayawardena, K.G.I.; Dassanayaka, V. & Vaughan, A. (1995). Kinetic and molecular differences in the amplified and non-amplified esterases from insecticide resistant and susceptible Culex quinquefasciatus mosquitoes. *The Journal of Biological Chemistry*, Vol.270, No.52, (December 1995), pp. 31124–31128, ISSN 1083-351X

Karunaratne, S.H.P.P. & Hemingway, J. (2001). Malathion resistance and prevalence of the malathion carboxylesterase mechanism in populations of mosquito vectors of disease in Sri Lanka. *Bulletin of the World Health Organization*, Vol.79, No.11, (November 2001), pp. 1060–1064, ISSN 1564-0604

Katz, T.M.; Miller, J.H. & Herbert, A.A. (2008). Insect repellents: Historical perspectives and new developments. *Journal of the American Academy of Dermatology*, Vol.58, No.5, (May 2008), pp. 865-871, ISSN 1097-6787

Khadjavi, A.; Giribaldi, G. & Prato, M. (2010). From control to eradication of malaria: the end of being stuck in second gear? *Asian Pacific Journal of Tropical Medicine*, Vol.3, No.5, (May 2010), pp. 412-420, ISSN 1995-7645

Kostaropoulos, I.; Papadopoulos, A.I.; Metaxakis, A.; Boukouvala, E. & Papadopoulou-Mourkidou, E. (2001). Glutathione S-transferase in the defence against pyrethroids in insects. *Insect Biochemistry and Molecular Biology*, Vol.31, No.4-5, (March 2001), pp. 313–319, ISSN 1879-0240

Lines, J.D.; Myamba, J. & Curtis, C.F. (1987). Experimental hut trials of permethrin-impregnated mosquito nets and curtains against malaria vectors in Tanzania. *Medical and Veterinary Entomology*, Vol. 1, No.1, (January 1987), pp. 37–51, ISSN 1365-2915

Lines, J. (1996). Mosquito nets and insecticides for net treatment: a discussion of existing and potential distribution systems in Africa. *Tropical Medicine & International Health*, Vol.1, No.5 , (October 1996), pp. 616–632, ISSN 1365-3156

Martinez Torres, D.; Chandre, F.; Williamson, M.S.; Darriet, F.; Bergé, J.B.; Devonshire, A.L.; Guillet, P.; Pasteur, N. & Pauron, D. (1998). Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector Anopheles gambiae s.s. *Insect Molecular Biology,* Vol.7, No.2, (May 1998), pp. 179–184, ISSN 1365-2583

Mathenge, E.M.; Gimnig, J.E.; Kolczak, M.; Ombok, M.; Irungu, L.W. & Hawley, W.A. (2001). Effect of permethrin-impregnated nets on exiting behaviour, blood feeding success, and time of feeding of malaria mosquitoes (Diptera: Culicidae) in Western Kenya. *Journal of Medical Entomology*, Vol.38, No.4, (July 2001), pp. 531–536, ISSN 0022-2585

Mbogo, C.N.M.; Baya, N.M.; Ofulla, A.V.O.; Githure, J.I. & Snow, R.W. (1996). The impact of permethrin-impregnated bednets on malaria vectors of the Kenyan coast. *Medical and Veterinary Entomology*, Vol.10, No.3, (July 1996), pp. 251–259, ISSN 1365-2915

McGlynn, K.A.; Quraishi, S.M.; Graubard, B.I.; Weber, J.P.; Rubertone, M.V. & Erickson, R.L. (2008). Persistent Organochlorine Pesticides and Risk of Testicular Germ Cell Tumors. *Journal of the National Cancer Institute*, Vol.100, No.9, (April 2008), pp. 663-671, ISSN 1460-2105

Ménard, R. (2005). Medicine: knockout malaria vaccine? *Nature*, Vol.13, No.433, (January 2005), pp. 113-114, ISSN 0028-0836

Mouches, C. ; Pasteur, N. ; Berge, J.B. ; Hyrien, O. ; Raymond, M. ; De Saint Vincent, B.R. ; de Silvestri, M. & Georghiou. G.P. (1986). Amplification of an esterase gene is responsible for insecticide resistance in a Californian Culex mosquito. *Science*, Vol.233, No.4765, (August 1986), pp. 778–780, ISSN 1095-9203

Mouchet, J. (1988). Mini review: agriculture and vector resistance. *Insect Science and Its Application* , Vol.9, No.3, (1988), pp. 297–302, ISSN 0191-9040

Munga, S.; Vulule, J. & Allan, R. (2009). Evaluation of insecticide treated wall lining materials used in traditional rural African houses, *Proceedings of 5th MIM Pan-African malaria conference*, Nairobi, Kenya, November 2009, Poster No. 734, pp. 200

Nauen, R. (2007). Insecticide resistance in disease vectors of public health importance. *Pest Management Science*, Vol.63, No.7, (July 2007), pp. 628-33, ISSN 1526-4998

N'Guessan, R.; Darriet, F.; Doannio, J.M.; Chandre, F. & Carnevale, P. (2001). Olyset net efficacy against pyrethroid resistant Anopheles gambiae and Culex quinquefasciatus after 3 years' field use in C te d'Ivoire. *Medical and Veterinary Entomology*, Vol.15, No.1, (March 2001), pp. 97–104, ISSN 1365-2915

O'Reilly, A.O.; Khambay, B.P.; Williamson, M.S.; Field, L.M.; Wallace, B.A. & Davies, T.G. (2006). Modelling insecticide-binding sites in the voltage-gated sodium channel. *The Biochemical Journal*, Vol.396, No.2, (June 2006), pp. 255–263, ISSN 1470-8728

Oppenoorth, F.J.; Van der Pas, L.J.T. & Houx, N.W.H. (1979). Glutathione S-transferases and hydrolytic activity in a tetrachlorovinphos- resistant strain of housefly and their influence on resistance. *Pesticide Biochemistry and Physiology*, Vol.11, No.1-3, (1979), pp. 176–188, ISSN 0048-3575

Osimitz, T.G. & Grothaus, R.H. (1995). The present safety assessment of DEET. *Journal of the American Mosquito Control Association*, Vol.11, No.2Pt2, (June 1995), pp.274-278, ISSN 8756-971X

Pates, H. & Curtis, C. (2005). Mosquito behavior and vector control. *Annual Review of Entomology*, Vol.50, (January 2005), pp. 53–70, ISSN 1545-4487

Penilla, R.P.; Rodriguez, A.D.; Hemingway, J.; Torres, J.L.; Solis, F. & Rodriguez, M.H. (2006). Changes in glutathione S-transferase activity in DDT resistant natural Mexican populations of Anopheles albimanus under different insecticide resistance management strategies. *Pest Management Science*, Vol.86, No.2, (October 2006), pp. 63–71, ISSN 1526-4998

Prapanthadara, L.; Hemingway, J. & Ketterman, A.J. (1995). DDT-resistance in Anopheles gambiae Giles from Zanzibar Tanzania, based on increased DDT-dehydrochlorinase activity of glutathione S-transferases. *Bulletin of Entomological Research*, Vol.85, No.2, (1995), pp. 267–274, ISSN 0007-4853

Prato, M.; Giribaldi, G.; Polimeni, M.; Gallo, V. & Arese, P. (2005). Phagocytosis of Hemozoin Enhances Matrix Metalloproteinase-9 Activity and TNF-alpha Production in Human Monocytes. Role of Matrix Metalloproteinases in the Pathogenesis of Falciparum Malaria. *The Journal of Immunology*, Vol.175, No.10, (November 2005), pp. 6436-6442, ISSN 1550-6606

Prato, M.; Gallo, V.; Giribaldi, G. & Arese, P. (2008). Phagocytosis of haemozoin (malarial pigment) enhances metalloproteinase-9 activity in human adherent monocytes: role of IL-1beta and 15-HETE. *Malaria Journal, Vol.7, No.157, (*August 2008), ISSN 1475-2875

Prato, M.; Giribaldi, G. & Arese, P. (2009). Hemozoin triggers tumor necrosis factor alpha-mediated release of lysozyme by human adherent monocytes: new evidences on leukocyte degranulation in P. falciparum malaria. *Asian Pacific Journal of Tropical Medicine*, Vol.2, No.3, (June 2009), pp. 35-40, ISSN 1995-7645

Prato, M.; Gallo, V. & Arese, P. (2010). Higher production of tumor necrosis factor alpha in hemozoin-fed human adherent monocytes is dependent on lipidic component of malarial pigment: new evidences on cytokine regulation in P. falciparum malaria.

Asian Pacific Journal of Tropical Medicine, Vol.3, No.2, (February 2010), pp. 85-89, ISSN 1995-7645

Prato, M.; Gallo, V.; Giribaldi, G.; Aldieri, E. & Arese, P. (2010). Role of the NF-kappaB transcription pathway in the hemozoin- and 15-HETE-mediated activation of matrix metalloproteinase-9 in human adherent monocytes. *Cellular Microbiology*, Vol.12, No.12, (December 2010), pp. 1780-1791, ISSN 1462-5822

Prato, M.; Gallo, V.; Valente, E.; Khadjavi, A.; Mandili, G. & Giribaldi, G. (2010). Malarial pigment enhances Heat Shock Protein-27 in THP-1 cells: new perspectives for in vitro studies on monocyte apoptosis prevention. *Asian Pacific Journal of Tropical Medicine*, Vol.3, No.12, (December 2010), pp. 934-938, ISSN 1995-7645

Prato, M. & Giribaldi, G. (2011). Matrix metalloproteinase-9 and haemozoin: wedding rings for human host and Plasmodium falciparum parasite in complicated malaria. *Journal of Tropical Medicine*, article ID 628435, (May 2011), ISSN 1687-9686

Prato, M.; D'Alessandro, S.; Van den Steen, P.E.; Opdenakker, G.; Arese, P.; Taramelli, D. & Basilico, N. (2011). Natural haemozoin modulates matrix metalloproteinases and induces morphological changes in human microvascular endothelium. *Cellular Microbiology*, Vol.13, No.8, (August 2011), pp. 1275-1285, ISSN 1462-5822

Prato, M. (2012). Role of human matrix metalloproteinases in malaria pathogenesis. In: *Malaria: Etiology, Pathogenesis and Treatments*, 2012. Nova Science Publishers, Inc. Hauppauge, ISBN 978-1-62100-363-2, NY, USA. In press.

Ranson, H. & Hemingway, J. (2004). Glutathione transferases, In: *Molecular Insect Science,* Gilbert, L.I.; Iatrou, K. & Gill, S. (Eds.), pp. 383-402, Elsevier Ltd, ISBN 0-44451-516-X, Oxford, UK

Ranson, H.; N'Guessan, R.; Lines, J.; Moiroux, N.; Nkuni, Z. & Corbel, V. (2011). Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? *Trends in Parasitology*, Vol.27, No.2, (February 2011), pp. 91-98, ISSN 1471-5007

Roberts, L. & Enserink, M. (2007). Did they really say… eradication? *Science*, Vol.318, No.5856, (December 2007), pp. 1544-1545, ISSN 1095-9203

Rogan, W.J. & Chen, A. (2005). Health risks and benefits of bis(4-chlorophenyl)-1,1,1-trichloroethane (DDT). *Lancet*, Vol.366, No.9487, (August 2005), pp. 763–773, ISSN 1474-547X

Rogier, C. & Hommel, M. (March 2011). Plasmodium life-cycle and natural history of malaria, In: Impact malaria sanofi Aventis' commitment, 9 March 2011, Available from http://www.impact-malaria.com/iml/cx/en/layout.jsp?cnt=2FAECA4C-CA72-4C97-9090-B725867E1579

Rooker, S.; Guillemaud, T.; Berge, J.; Pasteur, N. & Raymond, M. (1996). Coamplification of esterase A and B genes as a single unit in Culex pipiens mosquitoes. *Heredity*, Vol.77, No.Pt5, (November 1996), pp. 555–561, ISSN 1365-2540

Sadasivaiah, S.; Tozan, Y. & Breman, J.G. (2007). Dichlorodiphenyltrichloroethane (DDT) for Indoor Residual Spraying in Africa: How Can It Be Used for Malaria Control? *American Journal of Tropical Medicineand Hygiene,* Vol.77, No.6, (December 2007), pp. 249–263, ISSN 0002-9637

Santolamazza, F.; Calzetta, M.; Etang, J.; Barrese, E.; Dia, I.; Caccone, A.; Donnelly, M.J.; Petrarca, V.; Simard, F.; Pinto, J. & della Torre, A. (2008). Distribution of knock-

down resistance mutations in Anopheles gambiae molecular forms in west and westcentral Africa. *Malaria Journal*, Vol.7, No.74, (April 2008), ISSN 1475-2875

Sharp, B.L.; Ridl, F.C.; Govender, D.; Kuklinski, J. & Kleinschmidt, I. (2007). Malaria vector control by indoor residual insecticide spraying on the tropical island of Bioko, Equatorial Guinea. *Malaria Journal*, Vol.6, No.52, (May 2007), ISSN 1475-2875

Sinden, R.E. (2007). Malaria, mosquitoes and the legacy of Ronald Ross. *Bulletin of the World Health Organization*, vol.85, No.11, (November 2007), pp. 894–896, ISSN 1564-0604

Solberg, V.B.; Klein, T.A.; McPherson, K.R.; Bradford, B.A.; Burge, J.R. & Wirtz, R.A. (1995). Field evaluation of DEET and a piperidine repellent (A13-37220) against Amblyomma americanum (acari: Ixodidae). *Journal of Medical Entomology*, Vol.32, No.6, (November 1995), pp. 870-875, ISSN 0022-2585

Spinelli, J.J.; Ng, C.H.; Weber, J.P.; Connors, J.M.; Gascoyne, R.D.; Lai, A.S.; Brooks-Wilson, A.R.; Le, N.D.; Berry, B.R. & Gallagher, R.P. (2007). Organochlorines and risk of non-Hodgkin lymphoma. *International Journal of Cancer*, Vol.121, No.12, (December 2007), pp. 2767-2775 , ISSN 1097-0215

Stone, B.F. & Brown, A.W.A. (1969). Mechanisms of resistance to fenthion in Culex pipiens fatigans Wied. *Bulletin of the World Health Organization*, Vol.40, No.3, (March 1969), pp. 401–408, ISSN 1564-0604

Stump, A.D.; Atieli, F.K.; Vulule, J.M. & Besansky, N.J. (2004). Dynamics of the pyrethroid knockdown resistance allele in Western Kenyan populations of Anopheles gambiae in response to insecticide-treated bed net trials. *American Journal of Tropical Medicine and Hygiene*, Vol.70, No.6, (June 2004), pp. 591–596, ISSN 0002-9637

Takken, W. (2002). Do insecticide-treated bednets have an effect on malaria vectors? *Tropical Medicine and International Health*, Vol.7, No.12, (December 2002), pp. 1022–1030, ISSN 1365-3156

Tang, A.H. & Tu, C.P. (1994). Biochemical characterization of Drosophila glutathione S-transferases D1 and D21. The Journal of Biological Chemistry, Vol.269, No.45, (November 1969), pp. 27876–27884, ISSN 1083-351X

Teklehaimanot, A.; Sachs, J.D. & Curtis, C. (2007). Malaria control needs mass distribution of insecticidal bednets. *Lancet*, Vol.369, No.9580, (June 2007), pp. 2143–2146, ISSN 1474-547X

Toxipedia. (2011). Biological Properties of Insecticides, (11 May 2011), Available from http://toxipedia.org/display/toxipedia/Biological+Properties+of+Insecticides

Trieu, A.; Kayala, M.A.; Burk, C.; Molina, D.M.; Freilich, D.A.; Richie, T.L.; Baldi, P.; Felgner, P.L. & Doolan, D.L. (2011) Sterile protective immunity to malaria is associated with a panel of novel P.falciparum antigens. *Molecular and Cellular Proteomics*, in press, (May 2011), ISSN 1535-9484

Vaughan, A. & Hemingway, J. (1995). Mosquito carboxylesterase Est alpha 2(1) (A2). Cloning and sequence of the full-length cDNA for a major insecticide resistance gene worldwide in the mosquito Culex quinquefasciatus. *The Journal of Biological Chemistry*, Vol.270, No.28, (July 1995), pp. 17044–17049, ISSN 1083-351X

Vaughan, A.; Rodriguez, M. & Hemingway, J. (1995). The independent gene amplification of indistinguishable esterase B electromorphs from the insecticide resistant mosquito Culex quinquefasciatus. *The Biochemical Journal*, Vol.305, No.Pt2, (January 1995), pp. 651–658, ISSN 1470-8728

Vontas, J.G. ; Small, G.J. & Hemingway, J. (2001). Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in Nilaparvata lugens. *The Biochemical Journal*, Vol.357, No.Pt1, (July 2001), pp. 65–72, ISSN 1470-8728

WHO. (1993). Implementation of the global malaria control strategy, In: *Technical Report Series 839*, World Health Organization , ISBN 9-24120-839-2, Geneva, Switzerland.

WHO. (2005). Regional strategic framework for scaling up the use of insecticide-treated nets, In: *Insecticides treated materials*, n.d., Available from http://www.searo.who.int/LinkFiles/ Reports_MAL-239_&_VBC-87.pdf.

WHO. (2006a). WHO gives indoor use of DDT a clean bill of health for controlling malaria, In: *World Health Organization*, (20 September 2006), Available from http://www.who.int/mediacentre/ news/releases/2006/pr50/en/print.html.

WHO. (2006b). Indoor residual spraying, use of indoor residual spraying for scaling up global malaria control and elimination, In: *Indoor residual spraying,* World Health Organization, (11 December 2006), Available from http://www.who.int/malaria/vector_control/irs/en/

Zaim, M.; Aitio, A. & Nakashima, N. (2000). Safety of pyrethroid-treated mosquito nets. *Medical and Veterinary Entomology*, Vol.14, No.1, (March 2000), pp. 1–5, ISSN 1365-2915

5

Insecticides and Parasitoids

Toshiharu Tanaka and Chieka Minakuchi
Graduate School of Bio-Agricultural Sciences, Nagoya University, Japan

1. Introduction

More than 1,000,000 of insect species live on the earth with close association to each other. The population density of living organisms is regulated by abiotic and biotic factors during growth and development processes of each organism within some fluctuation, depressing the outbreak of some species. Abiotic factors like Flood or Dry involves in fluctuation of population. Biotic-regulation factors like deficiency of foods, predation and parasitism are important to depress the outbreak of population. In insects, parasitoids live over 200,000 species (Askew, 1971). Especially in agro-environment, planting monopolized by single crop in a wide area makes suitable condition for multiplication of some pest insect species and their outbreaks. Chemical control using pesticides for depression of pest population had been considered as one of better choice because of its immediate efficacy when outbreak happened. It has already clarified, however, that use of non-selective insecticides makes resurgence of insect pests caused by rapid decreasing of natural enemies. Agro-chemicals with selective toxicity have recently been developed, but it is not enough to examine their effects on natural enemies yet. To obtain agro-crops with secure and low price, we have to understand both specificities of natural enemies like parasitoids and of insecticides. Many parasitoid species works well to regulate the population density of pest insect in a well-conditioned cultivated space. Effective utilization of parasitoids and pesticides based on the various characteristics on each local region produce low density of pest population constantly. Simplified interaction between pest insects and natural enemies had made many unfortunate consequence of pest control like case of introduced natural enemies to invasion pests. Banker plants (Trap crops) are used for keeping the population of natural enemies permanently in constant density when natural enemies are multiplied and released artificially. It is developed as useful methods that 'companion plants' for supplying the foods like nector to natural enemies or 'refuge' as hiding place to prevent them from leaving and so on. However, devotion only to biological control is not adequate for regulation of pest population density corresponding to climate change year after year. When population of some pest insect breaks out to high density unexpected, natural enemies including parasitoid will lose to control for the population density of the pests. We will be forced to use chemical control temporarily. However, exclusive devotion to biological control with no pesticides or to chemical control ignoring biotic regulation seems not to produce the good results.

Although pest-control by IPM has been recommended recently, susceptibility of insecticides to parasitoids is not examined enough from various viewpoints. It is well known that parasitoids are one of important natural enemies to many pest species and are used

	Pest species	parasitoid	effects	references
Bt-toxin	*Choristoneura fumiferana*	*Apanteles fumiferanae*	high % of parasitism to aged host	Nealis, VG et al., 1992
	Prays olease	*Three species*	growth effect	Varlez, S et al. 1993
	Choristoneura fumiferana	*A. fumiferanae*	spray-timing	Cadogan, BL et al. 1995
	Plutella xylostella	*Diadegma insulare*	no effect	Ulpah, S et al. 1996
	P. xylostella	*D. insulare*	no effect	Xingeng, W & Shusheng, L. 1998
	P. xylostella	*Cotesia plutellae*	no effect on oviposition	Chilcutt, CF & Tabashnik, BE 1999
	Lymantria dispar	*Compsilura concinnata*	reduced parasitism	Erb, SL et al., 2001
	Liriomyza trifolii	*Diglyphus isaea, Dacnusa sibirica*	no effect	Ozawa A et al., 2001
	Mamestra brassicae	*Trichogramma dendrolimi*	no effect	Takada, Y et al., 2001
	Cnaphalocrocis medinalis	*Trichogramma chilonis*	no effect	Sehrawat S et al., 2002
	Pseudaletia unipuncta	*Gryptoapanteles militaris*	no effect	Schuler, TH et al., 2003
	rice pests	*parasitoid complex*	small effect	Schoenly, KG et al., 2003
	Helicoverpa armigera	*Microplitis mediator*	negative effect	Xu, YY et al., 2004
	Plutella xylostella	*C. plutellae*	small effect	Amend, J & Basedow, T 1997
	Plutella xylostella	*Diadegma insulare*	small effect	Jiang, YH et al., 2004
	Plutella xylostella	*Cotesia plutellae*	small effect	Sisterson, MS & Tabashnik, BE 2005
	Eldana saccharina, Busseola fusca, Sesamia calamistis	*Cotesia sesamiae*	positive effect	Bhatti, MA et al., 2005
	Plutella xylostella	*Diadegma fenestralis, Cotesia plutellae*	less effective	Bhardwaj, V et al., 2005
	Plutella xylostella	*Cotesia plutellae, Macromalon orientale, Diadromus collaris, Brachymeria excarinata*	small impact	Reyes, SG et al. 2005
	Tuta absoluta	*Trichogrammatoidea bactrae*	harmless	Riquelme Virgala, MB et al. 2006
	Anagasta kuehniella	*Thichogramma pratissolii*	no effect on wasp	Pratissoli, D. etal. 2006
	Anagasta kuehniella	*Thichogramma pratissolii, T. pretiosum*	no effect on wasp	Polanczyk, RA et al. 2006
	Chilo partellus	*Trichogramma chilonis*	no effect	Jalali, SK & Singh, SP 2006
	Anagasta kuehniella	*Thichogramma chilonis, Cheilomenes sexmaculata*	no effect	Basappa, H 2007
	Helicoverpa armigera	*Campoletis chlorideae*	significant reduction	Sharma, HC et al. 2008
	Plutella xylostella	*Diadegma semiclausum, Diadromus collaris*	better than chemicals	Nga, LT et al 2008
	Plutella xylostella, Pieris rapae	*Cotesia vestalis , Oomyzus sokolowskii Diadromus collaris C. glomeratus, C. rubecula, Pteromalus puparium*	weak impact	Furlong, MJ et al 2008
	Helicoverpa armigera	*Campoletis chlorideae*	high mortality	Mohan, M et al. 2008
	Plodia interpunctella	*Habrobracon hebetor*	fewer parasitoid emerged	Oluwafemi, AR et al. 2009
	Thaumetopoea pityocampa	*Exorista larvarum*	few effect	Marchetti E.et al. 2009
	Plutella xylostella	*Trichogrammatoidea bactrae*	no effect	Wang, D-S et al. 2010

Table 1-1. Effect of Bt-toxin on parasiotid species.

Bt-crops	Pest species	parasitoid	effect	ref
Bt-tobacco	*Heliothis virescens*	*Campoletis sonorensis, Cardiochiles nigriceps*	affect parasitoid larva of C. sonorensis	Johnson, MT、1997
Bt-corn	*Ostrinia nubilalis*	*Eriborus terebrans, Macrocentrus grandii Goidanich*	no difference	Orr, DB & Landis、DA, 1997
Bt-corn	*Ostrinia nubilalis*	*parasitoid community*	no difference	Lozzia, GC, 1999
Bt-potato	*Macrosiphum euphorbiae*	*Aphidius nigripes*	small effect	Ashouri, A et al. 2001
Bt-brassica	*Diaeretiella rapae*	*Myzus persicae*	no effect	Schuler, TH et al. 2001
Bt-corn	*Eoreuma loftini*	*Parallorhogas pyralophagus*	harmful	Bernal, JS et al. 2002
Bt-corn	*Ostrinia nubilalis*	*Lydella thompsoni, Pseudoperichaeta nigrolineata*	no difference	Bourguet, D et al. 2002
Bt-brassica	*Plutella xylostella*	*Cotesia plutellae*	no difference	Schuler, TH et al. 2003
Bt-corn	*Aphids*	*Aphidius ervi, A. rhopalosiphi, A. uzbekistanicus, Lysiphlebus testaceipes, Praon volucre*	no effect	Pons, X & Stary, O. 2003
Bt-cotton	*Pseudoplusia includens*	*Cotesia marginiventris, Copidosoma floridanum*	reduced longevity	Baur, ME & Boethel, DJ 2003
Bt-corn	*Ostrinia nubilalis*	*Lydella thompsoni*	no difference	Manachini, B 2003
Bt-rice	rice pests	paddy arthropod community	no effect	Liu, Z-C et al. 2003
Bt-brassica	*P. xylostella*	*C. plutellae*	no effect	Schuler, TH et al. 2004
Bt-corn	*Chilo partellus*	*C. flavipes*	harmful	Pruez, G & Dettner, K. 2004
Bt-rice	*Chilo suppressalis*	*Apanteles chilonis*	harmful	Jiang, Y-H et al. 2004
Bt-cotton	*Helicoverpa armigera*	*Trichogrammatoidea lutea, Telenomus ullyetti*	no influence	Mellet, MA et al.2004
Bt-corn	*Chilo partellus, its primary parasitoid Cotesia flavipes*	*Tetrastichus howardi (hyperparasitoid)*	harmful	Pruetz, G et al.2004
Bt-corn	*Micraspis discolor*	*Trichomma cnaphalocrosis*	no effect on behavior	Reyes, SG 2005
Bt-corn	european corn borer	arthropod community	less community disturbance	Dively, GP 2005
Bt-cotton	*Helicoverpa armigera*	*Micropilitis mediator*	minus effect	Liu, X-X et al. 2005
Bt-corn	*Spodoptera littoralis*	*Cotesia marginiventris*	negative effect	Vojtech, E et al. 2005
Bt-corn	*Diabrotica virgifera, Chaetocnema pulicaria, Rhopalosiphum maidis*	*ladybird beetles, damsel bugs, pirate bugs, flower flies, green lacewings, spidersMacrocentrus cingulum*	no adverse impact	Bhatti, MA et al. 2005
Bt-corn	*Ostrinia nubilalis*	*Coleomegilla maculata, Cycloneda munda, Orius insidiosus, Chrysoperla carnea, Macrocentrus cingulum*	some significantly affected	Pilcher, CD et al. 2005
Bt-cotton	*Helicoverpa armigera*	*parasitioid and pollinator*	parasitoid population decreased	Hofs, JL et al. 2005
Bt-corn	*Monolepta bifasciata*	*predatory beetles, lacewings, Trichogramma sp*	no effect	Reyes, SG & Jovillano-Mostoles, MDA 2005
Bt-corn	*Micraspis discolor*	*Trichomma cnaphalocrosis*	no difference	Reyes, SG. 2005
Bt potato	*Phthorimaea operculella*	*Apanteles subandinus, Micromus tasmaniae*	no effect	Davidson, MM et al. 2006
Bt-corn	*Spodoptera frugiperda*	*Cotesia marginiventris*	Bt was detected in parasitoid	Ramirez-Romero, R et al. 2007
Bt-potato, Bt-braccica	*Spodoptera litura, Helicoverpa armigera*	*Cotesia kazak, Meteorus pulchricornis*	a few impact	Walker, GP et al. 2007
Bt-corn	*Rhopalosiphum maidis*	*Cotesia marginiventris*	positive effect on performance of wasp	Faria, CA et al. 2007
Bt-cotton		parasitoid and predator	population density decreased	Han, L-Z et al. 2007
Bt-corn	*Spodoptera frugiperda*	*Campoletis sonorensis*	no effect on development	Sanders, CJ et al.2007
Bt-rice	*Sogatella furcifera, Nilaparvata lugens, Laodelphax striatellus*	*Cyrtorhinus lividipennis*	negative effect	Chen, M et al. 2007
Bt-cotton	*Helicoverpa armigera*	*predatory spiders, coccinellid, chrysopid*	siginificant reduction	Sharma, HC et al. 2007
Bt-cotton	*Helicoverpa armigera*	arthoropod community	no detrimental effect	Whitehouse, MEA et al. 2007
Bt-brassica	*Plutella xylostella*	*Diadegma insulare*	hazardless	Chen, M et al. 2008
Bt-brassica	*Pieris rapae*	*Pteromalus puparum*	significantly affected	Chen, M et al. 2008
Bt-brassica	*Plutella xylostella*	*Cotesia vestalis, Chrysoperla carnea*	trace of Bt	Wei, W et al. 2008
transgenic pine	*Pseudococremia suavis*	*Meteorus pulchricornis*	adverse effect	Barraclough, EI et al. 2009
Bt-chickpea	*Helicoverpa armigera*	*Campoletis chlorideae*	adverse effect	Dhillon, MK & Sharma, HC 2010
Bt-corn	*Spodoptera frugiperda*	*Cotesia marginiventris*	weak attractance	Desneux, N et al. 2010
Bt-corn		rove beetles as predator	no difference	Balog, A et al. 2010
Bt-cotton	*Spodoptera exigua, Heliothis armigera*	*Micropilitis tuberculifer*	no effect on behavior	Zhang, N et al. 2010

Table 1-2. Effect of Bt-crops on parasitoids.

extensively in biological and integrated pest control. More than 810 research papers related with insecticide and parasitoid in IPM have been accumulated during this decade from 2000 to 2010 for examining the impact of insecticides on introduced or native parasitoids and/or predators in laboratory condition or agro-fields, resulting that parasitoids are very high susceptibility to non-selective insecticides like pyrethroids, organophosphates, and carbamates except Bt toxin.

Recently Bt-toxin or Bt-transgenic crops have been developed and the susceptibility to parasitoids and/or predators (32 of Bt-toxin spray and 42 of Bt-crops in total 74 research papers, Table 1) was examined, resulting small impact on parasitoid and predator or on their communities.

Many reviews have already discussed about the side effect or the risk assessment of transgenic plants on non-target insects (Schuler et al. 1999; Groot & Dicke 2002; Dutton et al. 2003; Lövei & Arpaia 2005; Sisterson & Tabashnik 2005; Wolfenbarger et al., 2008; Lövei et al. 2009; Grzywacs et al. 2010; Gurr et al. 2010). However, severe problems have occurred also in Bt-transgenic crops that pest insects had gained the resistance to Bt-toxin just like development of the insecticide-resistance to many chemical insecticides. Approach like 'high dose/refuge strategy' (Chilcut & Tabashnik, 2004) or pyramid by expression of two genes have been tried to prolong the effectiveness of Bt-crops (Kumar et al. 2008, Ives et al., 2011). Although many chemical insecticides produced until present are toxic to natural enemies, we may be able to use them effectively by knowing the risk of chemical insecticides to maintain the predator and parasitoid communities sustainably.

In natural fields including agro-fields, parasitoids grow and develop mostly as eggs or larvae in/on their hosts and a few adult wasps stay with searching the hosts. Examination only on adult stage is insufficient for clarifying the susceptibility of parasitoid to insecticides. It is one of important points to examine the effect of chemical insecticides on the parasitized hosts in the developmental stages from oviposition to adult-emergence for evaluating the critical dosage to parasitoids. Effective usage of natural enemies like parasitoids in the agro-fields controlled by pesticides causes a decrease in the dosage of insecticides and brings agricultural crops with safety for human. Both ecological and physiological researches will be required for control of pest-population density. In this chapter, first as example, we tested effect of neonicotinoids on parasitoid along with the growth and development, for considering the characteristics of parasitoids.

1.1 Effect of insecticide on parasitoid

1.1.1 Direct and indirect effects of neonicotinoids on endoparasitoid along with the development

Recently although there are some researches published about the effect of neonicotinoids on egg parasitoids, there are a few papers on larval parasitoids (Table 2). These results showed variety from harmless to toxic impact.

However, the different results should be rearranged by difference of nutritional strategy between egg and larval parasitoids. Egg parasitoid ingests egg-yolk of the host soon after hatch as nutritional resource for growth, resulting become distended larval shape (Takada et al., 2000; Jarjees et al., 1998; Hutchison et al., 1990). Egg parasitoids are able to avoid the toxic effect of insecticides through the chorion of the host, using a protective role that is essential to normal development of the host embryo, and can circumvent the accumulation of toxic substance by sucking almost all egg-yolk from host egg at once after hatch. On the other hand, larval parasitoids have many chances to be exposed to insecticides during

category of parasitoid	parasitoid	target pest insect	Chemicals	effects	references
species richness	37 species of predators and parasitoids	leafhoppers, aphids, thrips and whiteflies	imidacloprid	no significant impact	Marquini, F. et al., 2002
egg parasitoid	*Aphytis melinus, Eretmocerus eremicus, Encarsia formosa, Gonatocerus ashmeadi*	California red scale and sweetpotato whitefly, glassy-winged sharpshooter,	acetamiprid	less toxic	Prabhaker, N., et al., 2007
	Trissolcus nigripedius	*Dolycoris baccarum*	thiamethoxam	less susceptible	Paine, TD, et al., 2011
	Aphytis melinus, Comperiella bifasciata, Rodolia cardinalis	*Aonidiella aurantii, Icerya purchasi, Panonychus citri*	imidacloprid	a little disruptive	Grafon-Cardwell, EE, et al., 2008
	aphlinid parasitoid	*Bemisia tabaci*	imidacloprid thiamethoxam	parasitism reduction	Naveed, M. et al., 2010
	Trichogramma pretiosum	*Anagasta kuehniella*	acetamiprid, imidacloprid	harmless	Carvalho, GA, et al., 2010
	Trichogramma minutum	*Acrobasis vaccinii*	thiacloprid acetamiprid	toxic	Wise, JC., et al., 2010
egg-, larval parasitoids	*Avetianella longoi, Syngaster lepidus*	-	imidacroprid	significantly lower survival	Lim, UT, 2008
egg-, egg-larval, larval parasitoids	*Trichogramma chilonis, Chelonus blackburni, Bracon hebetor*	*Gossypium hirsutum*	imidacloprid	moderate impact	Preetha, E et al., 2010
larval parasitoid	*Tiphia vernalis*	*Popillia japonica*	imidacloprid, thiamethoxam	adverse impact	Oliver, JB et al., 2005
	Diachasma alloeum	*Rhagoletis mendax, R. pomonella*	imidacloprid	adverse impact	Stelinski, LL et al., 2006
	Diadegma insulare, Oomyzus sokolowskii	*Plutella xylostella*	acetamiprid	less toxic	Cordero et al.,2007

Table 2. Effect of neonicotinoids on parasitoids.

developmental period (from egg to larval stages) and are dead together with the host when it is killed by insecticides, because Neonicotinoid have a strong effect on lepidopteran larvae. So in this chapter, to consider how to regulate the use of insecticide like neonicotinoid to larval parasitoid, it is necessary to examine the susceptibility of larval parasitoid to insecticide along with development. We use oriental armyworm *Mythimna separata* (Walker) as host and its endoparasitoid *Cotesia kariyai* (Watanabe) as a model system. *Mythimna separata* is a big pest for Poaceae plants and sometime make a big surge of population density. However, ecological population of *M. separata* is regulated with many kinds of parasitoids, major 5 species of endoparasitoids, *Campoletis chlorideae* Uchida, *Microplitis sp., C. kariyai, C. ruficrus* (Haliday), *Meteorus pulchricornis* (Wesmael), and an ectoparasitoid *Euplectrus separatae* Kamijo, and was normally kept low density under a local stable condition. *Cotesia kariyai* is a major gregarious endoparasitoid to oviposit from 30 to over 100 eggs in a *M. separata* host at once and can parasitize 2nd to 6th (last) host instar successfully (Tanaka et al., 1987).

1.1.2 Parasitoid wasps attack and oviposit the host *M. separata* treated previously with insecticides

To determine the sub-lethal dose activity of various neonicotinoids to unparasitized control, the unparasitized hosts 1, 2, 3 d after last ecdysis (D1L6, D2L6, D3L6, 6th larval stage is last instar) were used. Unparasitized hosts reach at the maximum weight 3 d after last ecdysis and become wandering stage to prepare pupation at D5L6. D2L6 larvae showed a low susceptibility to neonicotinoids, especially Thiamethoxam (Thm) and Dinotefran (Dnt), but high susceptibility to pyrethroid Permethrin (Per), organophosphate Fenitrothion (MEP), and Pyridalyl (Pyr) was observed, comparing to label rate (Table 3).

Insecticide	D1L6	D2L6	D3L6	Concentration used in each chemicals (ppm)	Label rate (ppm)
Acetamiprid (Act)	309.3	391.1	505.6	320	100
Thiamethoxam (Thm)	1163.8	1185.3	1158.3	400	50
Dinotefuran (Dnt)	1695.6	3116.6	1508.6	1200	100
Clothianidin (Clt)	204.6	1930.6	384.0	960	80
Thiacloprid (Thc)	702.5	640.2	780.3	640	150
Permethrin (Per)	160.7	57.5	247.2	24	100
fenitrothion (MEP)	192.0	169.2	131.7	24	500
Pyridalyl (Pyr)	0.7	4.0	0.3	0.8	100

Table 3. LC50 value (ppm) of various insecticide to unparasitized host *Mythimna separata.*

From these results, concentration of each insecticide treatment was determined. These values means different susceptibility of *M. separata* even on the same instar at sub-lethal dose, and it is hard to be generalized.

The emergence rate of parasitoid from host parasitized after insecticide treatment (post-treatment) informs us if parasitoids oviposit the host larvae treated by insecticides. Oviposition was performed within 2 hrs post-treatment of insecticide. Stinging behavior for oviposition was assured in every case. For example, Acetamiprid (Act), Thiacloprid (Thc) and Pyr treatments produced high larval emergence rate of parasitoid when parasitized post-treatment compared to pre-treatment, suggesting that oviposition was not disturbed by insecticide treatment (Fig. 1).

On the other hands, high pupation rate of hosts after Imidacloprid (Imd) treatment shows the possibility that parasitoid wasps may hesitate to inject the eggs though they stung the hosts. On the other hands, high emergence rate in insecticide treatment post-parasitization at sub-lethal dose means that the host or the parasitoid larvae possessed the detoxification ability to each insecticide and acquired some degree of tolerance to insecticides.

	LC50 (ppm)					
Chemicals*1	D1L6*2	D1L6P*3	D3L6	D3L6P	D5L6P	D 7 L6P
Act	309.3	608.4	505.6	176.1	786.9	377
Thm	1163.8	139.9	1158.3	40	49.9	436.3
Dnt	1695.6	1199	1508.6	1194.7	1102.6	1043.7
Clt	204.6	962	384.0	96	205.5	20.2
Thc	702.5	639.2	780.3	120	483.9	8563.3
Per	160.7	24	247.2	40.8	78.8	62.4
MEP	192.0	456	131.7	132	134.5	49.6
Pyr	0.7	3.5	0.3	4.4	3.9	2.5
*1 Act:Acetamiprid, Thm: Thiamethoxam, Dnt: Dinotefuran, Clt: Clothianidin, Thc: Thiacloprid, Per: Permethrin, MEP: fenitrothion, Pyr: Pyridalyl						
*2: day1- 6th instar of unparasitized control						
*3: day1-6th instar of parasitized host *Mythimna separata*						

Table 4. LC50 value of various neonicotinoids to unparasitized and parasitized hosts 1, 3, 5, 7 days after parasitization.

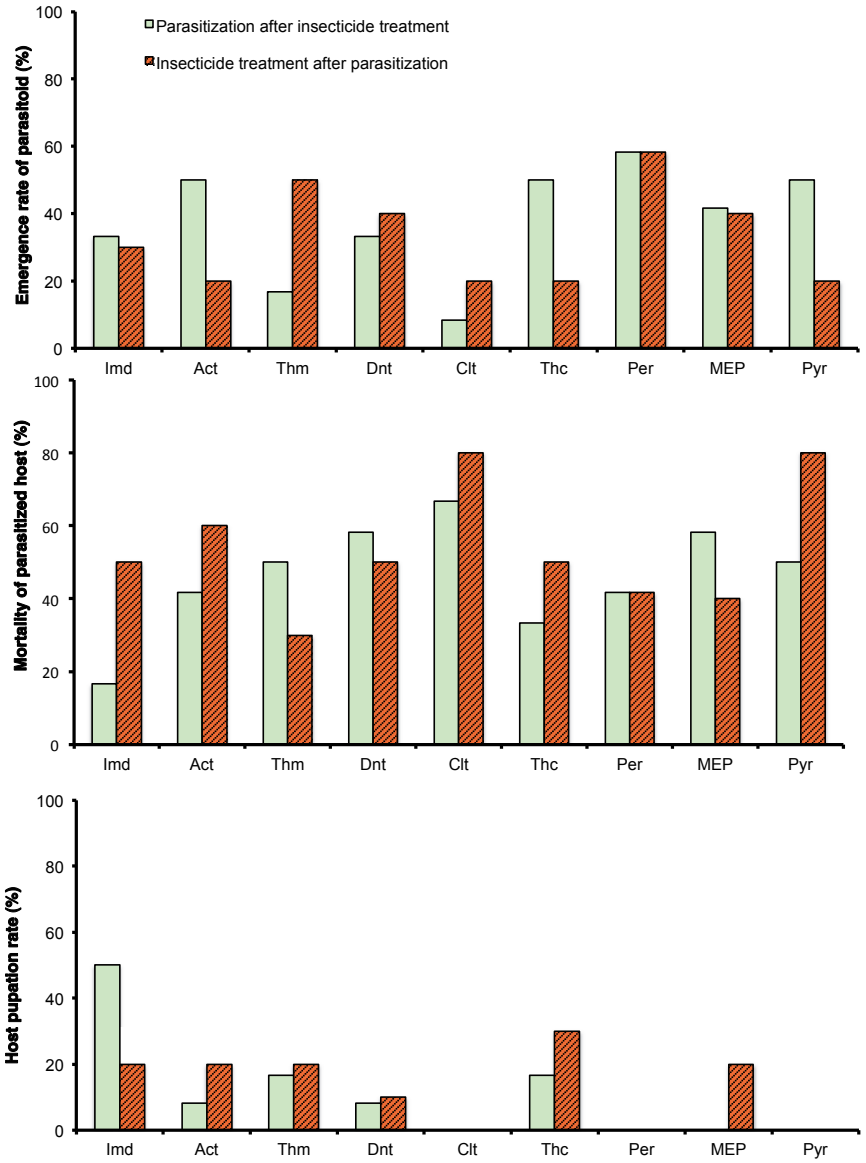


Fig. 1 Emergence rate of parasitoid larvae from the host treated by various insecticides at sub-lethal dose. Neonicotinoid insecticide treatment before parasitization made no impact on oviposition ef parasitoid wasp. The parasitoid larvae emerged from the host treated successfully when the parasitized hosts were not killed by insecticide treatment. Total number ef hosts treated with each insecticide was about 30 [ten for each, 3 replicates}. lmidacloprid (lmd), Acetamipriel (Act), Thiamethoxam (Thm), Dinotefuran (Dnt), Clothianidin (Git), Thiacloprid (The]. Permethrin (Per), fenitrothion (MEP), Pyridalyl (Pyr).

However, Clothianidin (Clt) treatment made low emergence rate of parasitoid causing by high mortality of host. Especially no emergence of parasitoid from parasitized hosts was observed also after day 3 post-parasitization (Fig. 2). Eggs of *C. kariyai* hatch and become 1st instar at 3.5 days after oviposition, and become 2nd instar on 5-6 days after oviposition. Insecticide treatment after parasitization was performed on each developmental point, on egg stage (day 1; D1), just before hatch (day 3; D3), on first instar (day 5; D5), on 2nd instar of parasitoid (day 7; D7). Parasitized hosts showed high susceptibility (meaning lower LC50 value) than that of unparasitized control, in treatment on every developmental days (Table 4), especially Clt treatment affect the larval emergence, meaning that parasitoid larva has no tolerance to Clt during larval stages. Further, Clt affect the adult eclosion heavily (Table 5).

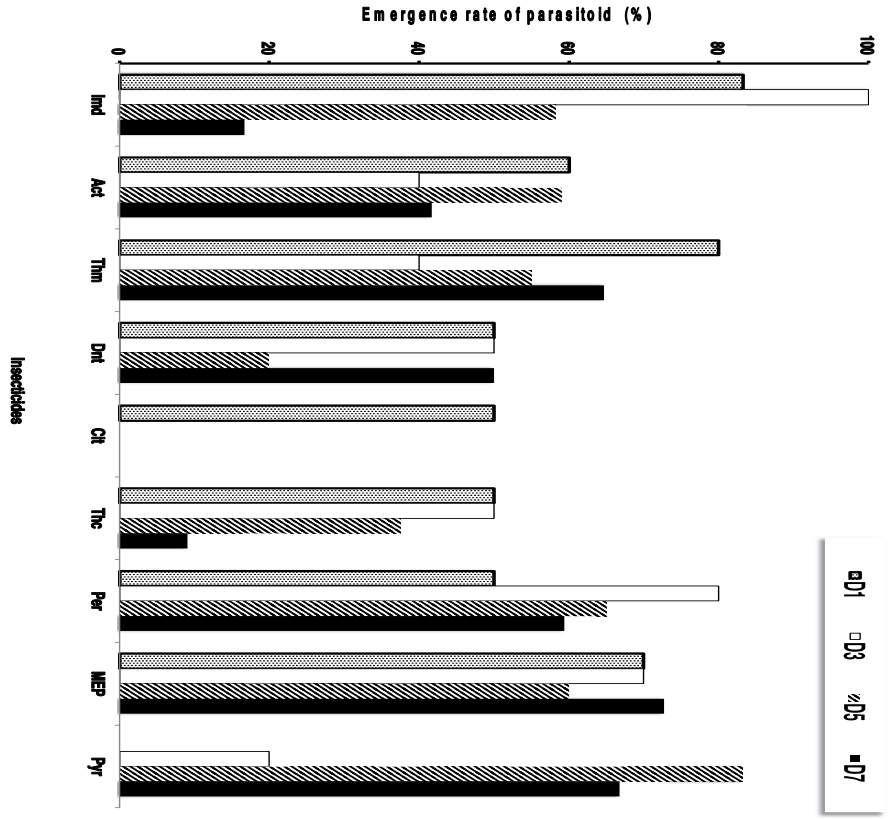


Fig. 2. Effect of treatment of insecticides along with development of the parasitoid on the parasitoid emergence rate sub-lethal dose. *Cotesia kariyai* hatches at 3.5 days post oviposition, 1st instar ecdyses to 2nd instar at 5 to 6 days, finally 3rd instars emerge from the host after ecdysis to 10 days after oviposition at 25 ± 1°C. Insecticide treatment was performed on 1, 3. 5. 7 days after parasitization. Total number of hosts treated with each insecticide was about 30 [ten per each, 3 replicates]. lmidalcoprid (lmd). Acetamiprid (Act), Thiamethoxam (Thm), Dinotefuran (Dnt). Clothianidin (Clt), Thiacloprid (Thc), Permethrin (Per), Fenitrothion (MEP), Pyridalyl (Pyr).

Insecticide	No of insects tested per each days	adult eclosion (%)			
		D1	D3	D5	D7
Imid	12	100	100	85.7	100
Act	12	100	100	100	80
Thm	12	100	75	81.8	59.1
Dnt	12	100	100	100	80
Clt	12	0	0	0	0
Thc	12	75	80	22.2	0
Per	12	100	100	100	90.9
MEP	12	100	100	83.3	100
Pyr	12	100	87.5	83.3	100

*1 Imd: Imidacloprid, Act:Acetamiprid, Thm: Thiamethoxam, Dnt: Dinotefuran, Clt: Clothianidin, Thc: Thiacloprid, Per: Permethrin, MEP: fenitrothion, Pyr: Pyridalyl

Table 5. Adult eclosion rate from cocoon of parasioid emerged from parasitized host treated by various insecticides.

For susceptibility of adult wasp to insecticide, ten female wasps was released in a 15 ml grass tube inside coated with active ingredients of various insecticide diluted in various concentration for 24 hrs with two replication (Table 6), resulting that parasitoid female wasps showed very high susceptibility to all insecticides. Even insecticides diluted than commercial label killed almost all wasps (Table 6).

Insecticide*1	Commercial Label recommended conc (ppm)	Concentration used (ppm)	Mortality of wasp (%)*2
Imd	100	60	80
Act	100	3.2	40
Thm	50	4	85
Dnt	100	12	80
Clt	80	9.6	100
Thc	150	12	95
Per	100	0.24	59
MEP	500	2.4	100
Pyr	100	0.08	20

*1 Imd: imidacloprid, Act:Acetamiprid, Thm: Thiamethoxam, Dnt: Dinotefuran, Clt: Clothianidin, Thc: Thiacloprid, Per: Permethrin, MEP: fenitrothion, Pyr: Pyridalyl

*2 Susceptible test for each insecticide was perofomed releasing 10 females in a 15 ml glass tube coated with active ingredients of each insecticide and dead wasps were counted 24 hrs later

Table 6. Susceptibility of parasitoid female wasp to neonicotinoid insecticides.

However, about 10 times diluted neonicotinoids like Thm, Clt, and Thc to LC50 value on D3L6 parasitized hosts made 80-100% mortality, in contrast to Permethrin diluted 50-100 times showed similar mortality. These results suggest that the neonicotinoids made slightly severe effect on larval parasitoid responsible for strong insecticidal potency to the death of lepidopteran hosts although they are less toxic than pyrethroids or organophosphates to parasitoid.

1.2 Parasitoid

Parasitoids are grouped in two categories as idiobiont and koinobiont based on nutritional strategy (Haeselbarth, 1979, Askew & Shaw, 1986). Parasitoids categorized as idiobiont that attack egg, pupal adult host stages, and paralyze or kill the hosts by venom preceding oviposition, thus develop in non-growing hosts and utilize the host resource existed at the time of parasitization for the growth and development. On the other hand, koinobiont can exploit the host resource increased after parasitization, because the parasitized hosts continue to grow and metamorphose during at least the initial stage of parasitism (Fig. 3). These include egg-larval and larval-pupal parasitoids or larval parasitoids that do not permanently paralyze their hosts at oviposition (Godfray, 1994).

1.2.1 Egg parasitoids as idiobiont

Idiobionts include many ectoparasitoids and egg or pupal endoparasitoids, and their venoms have characteristics to paralyse or kill the hosts and contain many kinds of enzymes to digestive most of host tissues with many variety (Moreau and Guillot, 2005). Venom of idiobionts as larval ectoparasitoids like Bracon spp. shows permanently paralyzing activity to the host (Beard, 1978, Quicke, 1997, Weaver et al. 1997). Venom is virulent and toxic potency to the host. Pupal ectoparasitoids also have to paralyze and fix the host to avoid consumption of food resource by growth of the host after parasitization with venom. On the other hands, *Nasonia vitripennis* as pupal endoparasitoid has non-paralysing venom that causes developmental arrest by 13 to 200.5 kDa proteins (Rivers et al., 2006), but venom shows PO (Phenol oxidase) activity and may induce apoptosis in host tissues (Abt & Rivers, 2007). *Mellitobia* wasp shows different mode of action in developmental arrest to different host species (Deyrup et al., 2006). These means that apoptotic tissues induced by venom are used for parasitoid development with time lag, with condition that their available resource is kept by developmental arrest. Idiobiont venom acts to arrest the host development and to ensure the food resource while preventing the unregulated decomposition by bacteria. Many kinds of venom in *Pimpla hypocondriaca* has already been reported and well reviewed by Moreau & Guillot (2005). In pupal endoparasitoid *Pimpla hypochondriaca*, many functional proteins in venom have been analysed; 28 k and 30 kDa proteins as serine protease (Parkinson et al. 2002a), 22 kDa as pimplin of paralytic peptide (Parkinson et al. 2002b), 39.9 kDa as reprolysin type metalloprotease (Parkinson et al. 2002c), 74 kDa with antibacterial and proteolytic activity (Dani et al., 2003).

Venom components of egg parasitoids is not clarified although a few case is analyzed; *Telenomus heliothidis* (Strand et al., 1983, 1986, Strand, 1986), *Trichogramma pretiosum* (Strand, 1986), *T. dendrolimi* (Takada et al., 2000), *T. australicum* (Jarjees & Merritt, 2004). Venoms (female-derived factor or acid gland) are injected into the host eggs with parasitoid egg and arrest the host development. Jarjees & Merritt (2004) suggested that venom was responsible for host die and degeneration of host tissues using sterile female.

Egg parasitoid ingests the host contents like yolk at once after hatch for growth and development (Takada et al., 2000) and consumes the contents of the host killed or decomposed by venom as nutritional resource for growth and development in the host. Rapid ingestion of the host yolk absorbed insecticides enhances the possibility of disturbance to the growth and development of the parasitoid. The difference of susceptibility of egg parasitoids to insecticides may be attributed from direct effect on the larval and pupal development and from the difference of food intake speed of the host contents, though it is further possibility that the residual insecticides outside of egg-shell disturb the emergence from host egg.

1.2.2 Larval endoparasitoid as koinobiont

Larval endoparasitoids have many chances to be affected during a long larval period indirectly through the host physiological action by insecticide. Koinobionts let the hosts survive and exploit nutrient from hosts during development of parasitoid associating with invasion in host hemocoel and are demanded both avoidance of host immune response and acquisition of nutrition from the host with minimal damage. Severe damage of the host during early development may lead to precocious death of the parasitoid. If they give severe damage to the hosts to get nutrients from hosts, parasitoids are exposed to risk for death.

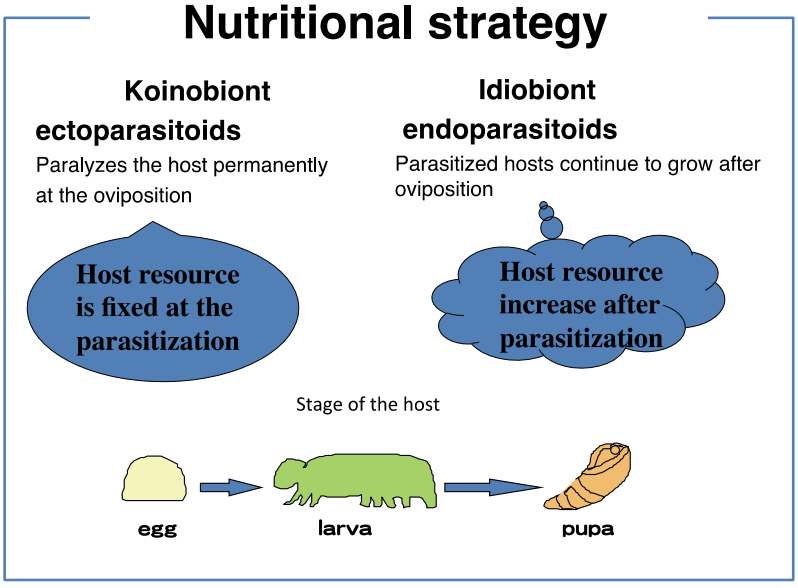


Fig. 3. Nutritional strategy of parasitioids.

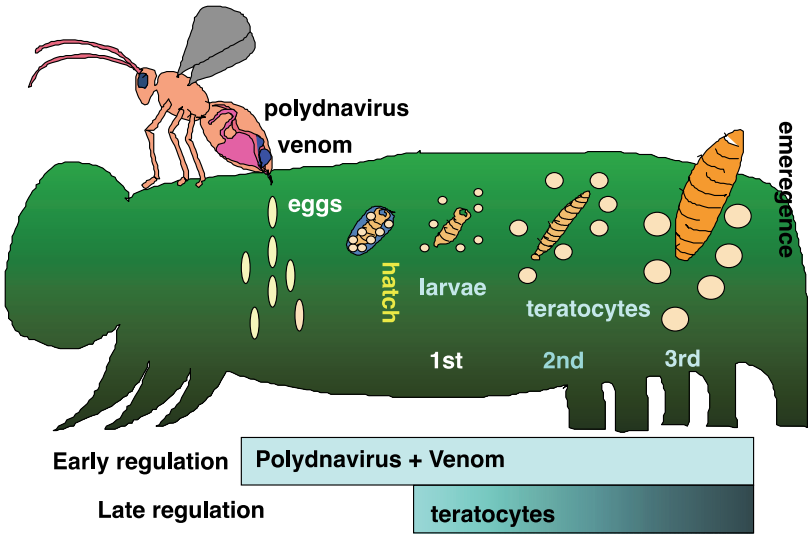


Fig. 4. Development of endoparasitoid and regulation factor of endoparasitoid on physiological state of the host. Polydnavirus (PDV) plus venom are injected into the host and regulate the physiological condition. Braconid endoparasitoids release their teratocytes which derived from the serosal cells of the egg after hatch and regulate the physiological condition during later stage.

Endoparasitoids develop the convenient tools like polydnaviruses (PDVs) plus venom and/or teratocytes to get nutrients without severe damage in evolutional process. Mutualistic relationship between PDVs and the endoparasitoids are estimated before about 70-73 ± 10 million years ago by calibration using fossil data (Whitfield, 2002, Drezen et al., 2003). Endoparasitoids seem to incorporate a nudivirus-related gene from ancestral Nudivirus and enable to produce the particles delivering in the host tissue cells for successful parasitism (Bézier et al., 2009a, b). PDV enables to regulate the physiological state of the host by penetration into each host tissue heterogeneously, especially in hemocytes and fat body. Viral genes expression alters the immune system and development of the host (Drezen et al., 2003, Beckage & Gelman, 2004, Kroemer & Webb, 2005, Webb & Strand, 2005, Gill et al., 2006, Asgari, 2006, Pennacchio & Strand, 2006, Kim et al., 2007). Hemocytes penetrated by PDV may lose ability to recognize and to encapsulate the foreign substances like eggs. Peptides or small proteins expressed from genes encoded in PDV play a role in physiological suppression of host immune response. Many suppression factors as PDV gene products are found. For example, protein tyrosine phosphatase (PTP) which known to play a critical role in the control of cellular events like proliferation, differentiation, and metabolism, and are a group of enzymes that remove phosphate groups from phosphorylated tyrosine residues on protein, then its expression affects the cellular PTP activity of the host (Espagne et al., 2004, Provost et al., 2004, Falabella et al., 2006, Gundersen-Rindal & Pedroni 2006, Ibrahim et al., 2007, Pruijssers & Strand, 2007, Ibrahin & Kim, 2008, Suderman et al., 2008, Shi et al., 2008), Cystatin which has inhibitory activity to cysteine proteases (Serbielle et al., 2008, Espagne et al., 2005), IkB-like (vankyrin) genes play a role in suppressing NF-kB activity in immune response (Kroemer & Webb, 2005, Bae

&Kim, 2009), and more Cysteine-rich domain products (Strand et al., 1997, Barandoc & Kim, 2009) and EP-1 like gene (Harwood & Beckage, 1994, Harwood et al., 1994, Kwon & Kim, 2008) including numerous hypothetical genes (Kroemer & Webb. 2004) may suppress the host immune response.

Venoms seem to change with evolution from ectoparasitoids to endoparasitoids (Whitfield, 2003), because venom may change from virulent action like killing the hosts to temperate action to lose toxic potency (Sclenke et al., 2007). Venoms of endoparasitoids contain many proteins in large molecular weight (Leluk et al., 1989) that lose the permanent paralytic function and promote of PDV expression in the host cells (Asgari, 2006).

Teratocytes are released and developed from serosal cell of parasitoid egg and produce some kind of regulatory protein along with the development (Fig.4). Endoparasitoids, on evolutionary process of having invaded from outside to inside, are required both to depress the host immune response specifically mentioned above and to get enough food and duration for growth and development at minimum damage to the host. Teratocytes play a role for extending larval stage of the host for getting enough nutrient required for their own growth and development. In case of Braconidae or Chalcidoidea, teratocytes function as one of factors to maintain the larval state (Dahlman et al., 2003). Elongation of larval state in parasitized hosts may increase the chance of contact with insecticides under natural condition. However, there is no information about detoxifying ability of teratocytes during late parasitism.

On the other hands, braconid endoparasitoids use teratocytes to take nutrients from host for avoiding severe damage to the host (Fig. 4). The most endoparasitoids seem to be assumed as hemolymph feeders (Thompson et al., 2001, 2002, Kaeslin et al., 2005), but In *C. kariyai-M. separata* association, second instars began to take fat body of host as food with help of teratocytes to ensure the big growth during 2nd instar stage (Nakamatsu et al, 2002, Tanaka et al. 2006). *Cotesia kariyai* also fed the host hemolymph as nutrient during first instar. Teratocytes attached on the surface and removed the outer membrane like cell matrix of the fat body with enzyme digestion locally, resulting that the second parasitoid larvae were easy to take the contents of the fat body as food. However, it is essential that the actin filaments in the fat body cells were broken previously by function of PDV plus venom (Tanaka et al., 2006). Although amount of consumption of the host fat body depend on the number of parasitoid larvae in a host, more than 100 parasitoid larvae consume almost all fat bodies (Nakamatsu & Tanaka, 2004). It was predicted that the larval endoparasitoids like *C. kariyai* might lower the susceptibility to insecticide during later parasitism by losing the fat body of the host.

1.2.3 Physiological regulation of endoparasitoid to insecticide

Koinobiont parasitoids that leave the host to continue growing after parasitization similar to unparasitized one are protected negatively through physiological action of the hosts from direct effect. Physiological milieu of the parasitized host is altered by PDV plus venom function from immediately after parasitization. Immune depression made us predict the lowering of resistance activity against the foreign substances penetrated into the body including xenobiotics and the detoxification ability of the host decreased with progressive ingestion of host fat body. However, in *Plutella xylostella- Cotesia vestalis (=plutellae*), Glutathion-s-transferase (GST) was enhanced the activity by PDV plus venom stimulation, because GST activity in egg stage was enhanced by oviposition or artificial injection of PDV

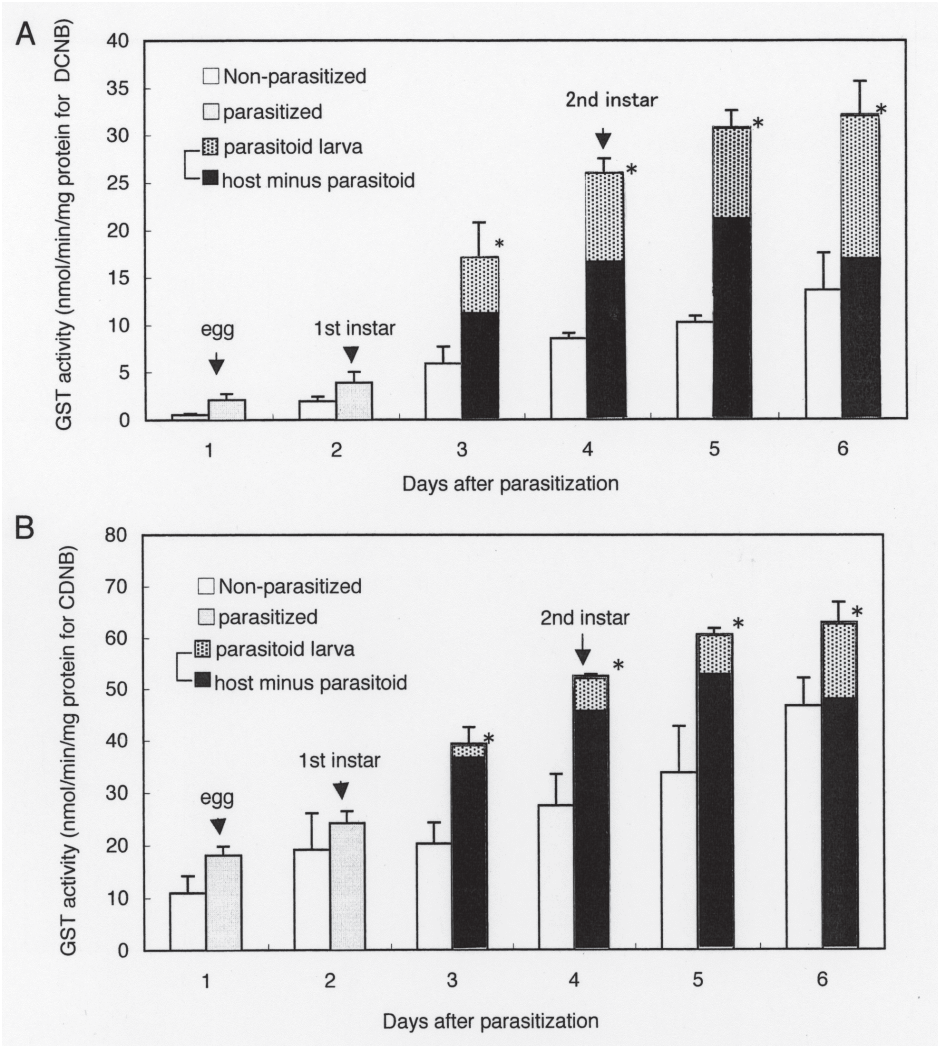


Fig. 5. Glutathion-s-transferase (GST) activity of the *Plutella xylostella* enhanced by parasitization of *Cotesia vestalis* (=*plutellae*). Data from Takeda et al. (2006). GST activity was measured with two enzyme substrates, individually (DCNB and CDNB). High GST activity of the hosts containing parasitioid larva was observed in later stage of parasitsm.

plus venom (Takeda et al. 2006). Especially during late stage of parasitization while parasitoid larva consumed the host fat body, a low susceptibility to organophosphate (diazinon and fenitrothion) was detected. It was clarified that enhancement of CYP and GST enzymes of both parasitoid larva in parasitized hosts and the host itself causes the low susceptibility to insecticides with high enzyme activity (Fig. 5 from Takeda et al., 2006). *Cotesia vestalis,* solitary endoparasitoid did not consume absolutely and remained the host fat body of the host. Further, endoparasitoid larva contributed to the detoxification of the

host after treatment of insecticide. Amount of fat body remained in the host after parasitization seemed to be determined by two factors, the degree of inhibition to the host growth after parasitization and amount of fat body consumed by the parasitoid larva. These suggested that the parasitized hosts are able to acquire the resistance to insecticides when parasitoids do not consume all the host fat body. The spraying of organophophates may make small impact on the surviving of parasitoids under agro-fields though the difference in susceptibility of parasitoids is not examined.

2. Conclusion

Parasitoids have different nutritional strategy. This difference seems to affect the susceptibility to insecticide. Idiobiont like egg parasitoid can utilize the dead host as nutritional resource. Normally idiobiont parasitoids kill or paralyze the host and stop the development of the host using venom. Many reports inform us a little effect of insecticides on the egg parasitoids. If insecticide hard to penetrate inside the host egg, parasitoid wasps can emerge from the parasitized eggs except that residual effect on the egg-shell kill the wasps at the emergence. On the other hands, koinobiont parasitoids utilize the host that continues to grow after parasitization, and are kept under physiological depression, especially in immune response by PDV plus venom. These mean the high susceptibility to insecticides during larval development. After all, larval parasitoids cannot develop in and emerge from hosts killed by insecticide treatment during their development even if the parasitoid larvae have resistance against the pesticide chemicals. Sub-lethal dose did not make severe effect on emergence rate of parasitoid even when insecticide treatment was performed during late parasitism except some neonicotinoids, though the susceptibility of the hosts treated with insecticides before parasitization or of the hosts treated with insecticides after parasitization along with growth and development was different between insecticides. On the other hands, parasitoid wasps had a high susceptibility to insecticides. When the insecticide spray in the agro-fields should be performed using place to escape for wasps like refuge, companion or banker plants. If transgenic crops will be used with methods or techniques that constrain the development of resistance strain, it may be valid and useful to depress the pest insect population. The parasitoid larvae were successfully emerged from the parasitized hosts at sub-lethal dose anytime during larval development, though the emergence rate is low. The parasitoids emerged from the hosts may lead to the potential to regulate the population density of pest insect.

3. Acknowledgment

We thank Zen-Noh Co. Ltd and Sumitomo Chemical Co. Ltd for providing insecticides. This work was supported in a part by a grant-in-aid for Scientific Research Work of Mistry of Education, Science and Culture, Japan.

4. References

Abt, M; Rivers, DB (2007) Characterization of phenoloxidase activity in venom from the ectoparasitoid *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae). *Journal of Invertebrate Pathology* 94, 108-118

Amend, J.; Basedow, T. (1997) Combining release/establishment of *Diadegma semiclausum* (Hellen) (Hym., Ichneumonidae) and Bacillus thuringiensis Berl. for control of

Plutella xylostella (L.) (Lep., Yponomeutidae) and other lepidopteran pests in the Cordillera Region of Luzon (Philippines). *Journal of Applied Entomology* 121, 337-342

Asgari, S (2006) Venom proteins from polydnavirus-producing endoparasitoids: their role in host-parasite interactions. *Archives of Insect Biochemistry and Physiology* 61, 146-156.

Ashouri, A; Michaud, D; Cloutier, C. (2001) Recombinant and classically selected factors of potato plant resistance to the Colorado potato beetle, *Leptinotarsa decemlineata*, variously affect the potato aphid parasitoid Aphidius nigripes. *BioControl* (Dordrecht) 46, 401-418

Ashouri, A. (2004) Transgenic-Bt potato plant resistance to the Colorado potato beetle affect the aphid parasitoid *Aphidius nigripes*. *Communications in Agricultural and Applied Biological Sciences* 69, 185-189

Askew, RR (1971) Parasitic hymenoptera *in* Parasitic Insects, p.113-184, Heineman Educational Books, London, Wood ward (Bath) Ltd.

Askew, RR, Shaw, MR (1986) Parasitoid communities: Their size, structure and development. *in* JK Waage and D. Greathead (eds.). *Insect Parasitoids*, pp. 225-264. Academic Press, London.

Bae, S; Kim, Y (2009) IkB genes encoded in *Cotesia plutellae* bracovirus suppress an antiviral response and enhance baculovirus pathogenicity against the diamondback moth, *Plutella xylostella*. *Journal of Invertebrate Pathology* 102, 79-87.

Balog, A; Kiss, J; Szekeres, D; Szenasi, A; Marko, V. (2010) Rove beetle (Coleoptera: Staphylinidae) communities in transgenic Bt (MON810) and near isogenic maize. *Crop Protection* 29, 567-571

Barandoc, K; Kim, Y (2009) A technique of segment expression and RNA interference (SERI) reveals a specific physiological function of a cysteine-rich protein gene encoded in *Cotesia plutellae* bracovirus. *Journal of Microbiology and Biotechnology* 19, 610–615

Barraclough, EI; Burgess, EPJ; Philip, BA; Wohlers, MW; Malone, LA (2009) Tritrophic impacts of Bt-expressing transgenic pine on the parasitoid *Meteorus pulchricornis* (Hymenoptera: Braconidae) via its host *Pseudocoremia suavis* (Lepidoptera: Geometridae). *Biological Control* 49. 192-199

Basappa. H (2007) Toxicity of biopesticides and synthetic insecticides to egg parasitoid, *Trichogramma chilonis* Ishii and coccinellid predator, *Cheilomenes sexmaculata* (Fabricius). *Journal of Biological Control* 21,31-36

Baur, ME; Boethel, DJ (2003) Effect of Bt-cotton expressing Cry1A(c) on the survival and fecundity of two hymenopteran parasitoids (Braconidae, Encyrtidae) in the laboratory. *Biological Control* 26, 325-332

Beard, RL (1978) *Venom of Braconidae*. *in* Handbook of Experimental Pharmacology 48, pp. 773-800, Springer-Verlag, New York.

Beckage, NE; Gelman, DB (2004) Wasp parasitoid disruption of host development: implications for new biologically based strategies for insect control. *Annual Review of Entomology* 49, 299-330

Bell, HA; Kirkbride-Smith, AE; Marris, GC; Edwards, JP (2004) Teratocytes of the soliary endoparasitoid *Meteorus gyrator* (Hymenoptera: Braconidae): morphology, numbers and possible functions. *Physiological Entolomogy* 29, 335-343.

Bernal, JS; Griset, JG; Gillogly, PO (2002) Impacts of developing on Bt maize-intoxicated hosts on fitness parameters of a stem borer parasitoid. *Journal of Entomological Science* 37, 27-40

Bézier, A; Herbinière, J; Lanzrein, B; Drezen, JM (2009a) Polydnavirus hidden face: The genes producing virus particles of parasitic wasps. *Journal of Invertebrate Pathology* 101 (2009) 194–203

Bézier, A; Annaheim, M; Herbinière, J; Wetterwald, C; Gyapay, G; Bernard-Samain, S; Wincker P; Roditi, I; Heller, M; Belghazi, M; Pfister-Wilhem, R; Periquet, G; Dupuy, C; Huguet, E; Volkoff, AN; Lanzrein, B; Drezen, JM (2009b) Polydnaviruses of braconid wasps derive from an ancestral nudivirus. *Science* 323, 926-930

Bhardwaj, V; Devi, N; Raj, D (2005) Toxicity of some insecticides and biopesticides to *Diadegma fenestralis* Himage and *Cotesia plutellae* (Kurdyumov), two parasitoids of diamondback moth. *Journal of Entomological Research* (New Delhi) 29, 27-30

Bhatti, MA; Duan, J; Head, GP; Jiang, C; McKee, MJ; Nickson, TE; Pilcher, CL; Pilcher, CD (2005) Field evaluation of the impact of corn rootworm (Coleoptera : Chrysomelidae)-protected Bt corn on foliage-dwelling arthropods. *Environmental Entomology* 34, 1336-1345

Bourguet, D; Chaufaux, J; Micoud, A; Delos, M; Naibo, B; Bombarde, F; Marque, G; Eychenne, N; Pagliari, C (2002) *Ostrinia nubilalis* parasitism and the field abundance of non-target insects in transgenic *Bacillus thuringiensis* corn (Zea mays). *Environmental Biosafety Research* 1, 49-60

Cadogan, BL; Nealis, VG; Van Frankenhuyzen, K (1995) Control of spruce budworm (Lepidoptera: Tortricidae) with *Bacillus thuringiensis* applications timed to conserve a larval parasitoid. *Crop Protection* 14, 31-36

Carvalho, GA; Godoy, MS; Parreira, DS; Lasmar, O; Souza, JR; Moscardini, VF (2010) Selectivity of growth regulators and neonicotinoids for adults of *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae). *Revista Colombiana de Entomologia* 36, 195-20

Chen, M; Liu, Z-C; Ye, G-Y; Shen, Z-C; Hu, C; Peng, Y-f; Altosaar, I; Shelton, AM (2007) Impacts of transgenic cry1Ab rice on non-target planthoppers and their main predator *Cyrtorhinus lividipennis* (Hemiptera: Miridae) - A case study of the compatibility of Bt rice with biological control. *Biological Control* 42, 242-250

Chen, M; Zhao, J-Z; Collins, HL; Earle, ED; Cao, J; Shelton, AM (2008) A critical assessment of the effects of Bt transgenic plants on parasitoids. *PLoS One* 3, e2284

Chen, M; Zhao, JZ; Shelton, AM; Cao, J; Earle, ED (2008) Impact of single-gene and dual-gene Bt broccoli on the herbivore *Pieris rapae* (Lepidoptera: Pieridae) and its pupal endoparasitoid *Pteromalus puparum* (Hymenoptera : Pteromalidae). *Transgenic Research* 17, 545-555

Chilcutt, CF; Tabashnik, BE (1999) Effects of Bacillus thuringiensis on adults of *Cotesia plutellae* (Hymenoptera: Braconidae), a parasitoid of the Diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae). *Biocontrol Science and Technology* 9, 435-440

Chilcutt, CF.; Tabashnik, BE (2004) Contamination of refuges by *Bacillus thuringiensis* toxin genes from transgenic maiz. *Proceedings of the National Academy of Sciences of the United States of America,* 101, 7526-7529.

Cordero, RJ; Bloomquist, JR; Kuhar, TP (2007) Susceptibility of two diamondback moth parasitoids, *Diadegma insulare* (Cresson) (Hymenoptera; Ichneumonidae) and *Oomyzus sokolowskii* (Kurdjumov) (Hymenoptera; Eulophidae), to selected commercial insecticides. *Biological Control* 42, 48-54

Dahlman, DL; Rana, RL; Schepers, EJ; Schepers, T; DiLuna, FA; Webb BA (2003) A teratocyte gene from a parasitic wasp that is associated with inhibition of insect growth and development inhibits host protein synthesis. *Insect Molecular Biology* 12, 527–534

Dani, MP; Richards, EH; Isaac, RE; Edwards, JP (2003) Antibacterial and proteolytic activity in venom from the endoparasitic wasp *Pimpla hypochondriaca* (Hymenoptera: Ichneumonidae). *Journal of Insect Physiology* 49,945-954.

Davidson, MM; Butler, RC; Wratten, SD; Conner AJ (2006) Impacts of insect-resistant transgenic potatoes on the survival and fecundity of a parasitoid and an insect predator. *Biological Control* 37, 224-230

Desneux, N; Ramirez-Romero, R; Bokonon-Ganta, AH; Bernal, JS (2010) Attraction of the parasitoid *Cotesia marginiventris* to host (Spodoptera frugiperda) frass is affected by transgenic maize. *Ecotoxicology* 19, 1183-1192

Deyrup, LD; Rivers, DB; Matthews, RW (2006) Venom from the ectoparasitic wasp *Mellitobia digitata* (Hymenoptera: Eulophidae) induces paralysis and developmental delay in natural and factitious hosts. *Annals of the Entomological Society of America* 99, 1199-1205.

Dhillon, MK; Sharma, HC (2010) Chickpea-mediated effects of *Bacillus thuringiensis* on *Helicoverpa armigera* and its larval parasitoid,*Campoletis chlorideae*. *Journal of Applied Entomology* 134, 682-693

Dively, GP (2005) Impact of transgenic VIP3A x Cry1Ab lepidopteran-resistant field corn on the nontarget arthropod community. *Environmental Entomology* 34, 1267-1291

Drezen, JM; B. Provost, B; Espagne, E Cattolico, L; Dupuy, C; Poirié, M; Periquet, G; Huguet, E (2003) Polydavirus genome: integrated vs. free virus. *Journal of Insect Physiology* 49, 407-417

Dutton, A.; Romeis, J; and Franz Bigler, F (2003) Assessing the risks of insect resistant transgenic plants on entomophagous arthropods: Bt-maize expressing Cry1Ab as a case study. *BioControl* 48: 611–636

Erb, SL; Bourchier, RS; van Frankenhuyzen, K; Smith, SM (2001) Sublethal effects of *Bacillus thuringiensis* Berliner subsp. kurstaki on *Lymantria dispar* (Lepidoptera: Lymantriidae) and the tachinid parasitoid *Compsilura concinnata* (Diptera: Tachinidae). *Environmental Entomology* 30, 1174-1181

Espagne,E; Catherine Dupuy, C; Huguet, E; Cattolico, L; Prevost, B; Martins, N; Maryl`ene Poirié, M; Periquet, G; Drezen, JM (2004) Genome sequence of a polydnavirus: insights into symbiotic virus evolution. *Science* 306, 286-289

Espagne,E; Douris, V; Lalmanach, G; Provost, B; Cattolico, L; Lesobre, J; Kurata, S; Iatrou, K; Drezen, JM; Huguet, E (2005) A virus essential for insect host-parasite interactions encodes cystatins. *Journal of Virology* 79, 9765–9776

Falabella, F; Caccialupi, P; Varricchio, P; Carla Malva, C; Pennacchio, F (2006) Protein tyrosine phosphatases of *Toxoneuron nigriceps* Bracovirus as potential disrupters of host prothoracic gland function. *Archives of Insect Biochemistry and Physiology* 61:157-169

Faria, CA; Waeckers, FL; Pritchard, J; Barrett, DA; Turlings, TCJ (2007) High susceptibility of Bt maize to aphids enhances the performance of parasitoids of lepidopteran pests. *PLoS One* 2, e600

Furlong, MJ; Ju, KH; Su, PW; Chol, JK; Il, RC; Zalucki, MP (2008) Integration of endemic natural enemies and *Bacillus thuringiensis* to manage insect pests of Brassica crops in North Korea. *Agriculture Ecosystems & Environment* 125, 223-238

Gao, M-Q; Hou, S-P; Pu, D-Q; Shi, M; Ye, G-Y; Chen, X-X (2010) Multi-generation effects of Bt rice on *Anagrus nilaparvatae*, a parasitoid of the nontarget pest *Nilapavarta lugens*. *Environmental Entomology* 39, 2039-2044

Geng, J-H; Shen, Z-R; Song, K; Zheng, L (2006) Effect of pollen of regular cotton and transgenic Bt plus CpTI cotton on the survival and reproduction of the parasitoid wasp *Trichogramma chilonis* (Hymenoptera: Trichogrammatidae) in the laboratory. *Environmental Entomology* 35, 1661-1668

Gill, T; Angelika Fath-Goodin, A; Maiti, II; Webb, BA (2006) Potential uses of cys-motif and other polydnavirus genes in biotechnology. *Advances in Virus Research* 68, 393-426

Godfray, HCJ (1994) Parasitoids: *Behavioral and Evolutionary Ecology*. Princeton Univeristy Press, Princeton.

Gorecka, J; Godzina, M; Dqbrowski, ZT (2008) Effect of Bt maize MON 810 expressing Cry 1 Ab toxin on *Aphidius colemani* in tritrophic plant - herbivore - parasitoid system. *Journal of Plant Protection Research* 48, 129-136

Grafton-Cardwell, EE; Lee, JE; Robillard, SM; Gorden, JM (2008) Role of imidacloprid in integrated pest management of California citrus. *Journal of Economic Entomology* 101, 451-460

Groot, A.T. and M. Dicke (2002) Insect-resistant transgenic plants in a multi-trophic context. *The Plant Journal* 31: 387–406.

Grzywacz, D; Rossbach, A; Rauf, A; Russell, DA;Srinivasan, R; Shelton, AM (2010) Current control methods for diamondback moth and other brassica insect pests and the prospects for improved management with lepidopteran-resistant Bt vegetable brassicas in Asia and Africa. *Crop Protection* 29, 68–79

Gundersen-Rindal, DE; Pedroni, MJ (2006) Characterization and transcriptional analysis of protein tyrosine phosphatase genes and an ankyrin repeat gene of the parasitoid Glyptapanteles indiensis polydnavirus in the parasitized host. *Journal of General Virology* 87, 311–322

Gurr, GM; Liu, J; Read DMY; Catindig, JLA; Cheng, JA; Lan, LP; Heong, KL (2010) Parasitoids of Asian rice planthopper (Hemiptera: Delphacidae) pests and prospects for enhancing biological control by ecological engineering. *Annals of Applied Biology* 158, 149–176

Haeselbarth, E (1979) Zur Parasitierrung der Puppen von Forleule (Panolis flammea (Schiff.)), Kiefernspanner (Bupalus piniarius (L.)) und Heidelbeerspanner (Boarmia biostortana (Goeze)) in bayerischen Kiefernwäldern. *Zeitschrift für Angewandte Entomologie* 87, 186-202.

Han L-Z; Bai S-X; Zhao J-Z; Wang Z-Y; Wu K-M (2007) Progress in ecological biosafety of insect-resistant transgenic cotton and corn in relation to arthropods. *Acta Entomologica Sinica* 50, 727-736

Harwood, SH; Beckage, NE (1994) Purification and characterization of an early-expressed polydnavirus-induced protein from the hemolymph of *Manduca sexta* larvae parasitized by *Cotesia congregata*. *Insect Biochemistry and Molecular Biology* 24, 685-698

Harwood, SH; Grosovsky, AJ; Cowles, EA; Davis, JW; Beckage, NE (1994) An abundantly expressed hemolymph glycoprotein isolated from newly parasitized *Manduca sexta* larvae is a polydnavirus gene product. *Virology* 205, 381-392

Haseeb, M; Liu, T-X; Jones, WA (2004) Effects of selected insecticides on Cotesia plutellae, endoparasitoid of *Plutella xylostella*. *BioControl* (Dordrecht) 49, 33-46

Hofs, J-L; Schoeman, At; Mellet, M; Vaissayre, M (2005) Impact of genetically-modified cotton plants of the biodiversity on entomologic fauna: The case of Bt cotton in South Africa. *International Journal of Tropical Insect Science* 25, 63-72

Hutchison, WD; Moratorio, M; Martin, JM (1990) Morphology and biology of *Trichogrammatoidea bactorae* (Hymenoptera: Trichogrammatidae), imported from Australia as a parasitoid of pink bollworm (Lepidoptera: Gelechiidae) eggs. *Annals of the Entomological Society of America* 83, 46-54.

Ibrahim, AMA; Choi、JY; Yeon Ho Jeb; Kim, Y (2007) Protein tyrosine phosphatases encoded in *Cotesia plutellae* bracovirus: Sequence analysis, expression profile, and a possible biological role in host immunosuppression. *Developmental and Comparative Immunology* 31, 978-990

Ibrahim AMA; Kim, Y (2008) Transient expression of protein tyrosine phosphatases encoded in *Cotesia plutellae* bracovirus inhibits insect cellular immune responses. *Naturwissenschaften* 95, 25–32

Ives, AR; Glaum, PR; Ziebarth, NL; Andow, DA (2011) The evolution of resistance to two-toxin pyramid transgenic crops. *Ecological Applications* 21, 503-515

Jacas, JA; Pen, JE; Duncan RE (2009) Morphology and development of the immature stages of *Brachyufens osborni* (Hymenoptera: Trichogrammatidae), an egg parasitoid of broad-nosed weevil species (Coleoptera: Curculionidae). *Annals of the Entomological Society of America* 102, 112-118

Jalali, SK; Singh, SP (2006) Biological control of *Chilo partellus* using egg parasitoid *Trichogramma chilonis* and *Bacillus thuringiensis*. *Indian Journal of Agricultural Research* 40, 184-189

Jarjees, E; J. Merritt DJ; Gordh, G (1998) Anatomy of the mouthparts and digestive tract during feeding in larvae of the parasitoid wasp *Trichogramma australicum* Girault (Hymenoptera: Trichogrammatidae). *International Journal of Insect Morphology and Embryology* 27, 103-110.

Jarjees, EA ; Merritt, DJ (2004) The effect of parasitization by *Trichogramma australicum* on *Helicoverpa armigera* host eggs and embryos. *Journal of Invertebrate Pathology* 85, 1-8

Jiang, Y-H; Fu, Q; Cheng, J-A; Ye, G-Y; Bai, Y-Y; Zhang, Z-t (2004) Effects of transgenic Bt rice on the biological characteristics of *Apanteles chilonis* (Munakata) (Hymenoptera: Braconidae). *Acta Entomologica Sinica* 47, 124-129

Johnson, MT (1997) Interaction of resistant plants and wasp parasitoids of tobacco budworm (Lepidoptera: Noctuidae). *Environmental Entomology* 26, 207-214

Kaeslin, M; Pfister-Wilhelm R; Lanzrein , B (2005) Influence of the parasitoid *Chelonus inanitus* and its polydnavirus on host nutritional physiology and implications for parasitoid development. *Journal of Insect Physiology* 51, 1330–1339

Kim, Y; Choi, JY; Je, YH (2007) *Cotesia plutellae* bracovirus genome and its function in altering insect physiology. *Journal of Asia-Pacific Entomology* 10, 181-191

Kroemer, JA; Webb, BA (2004) Polydnavirus genes and genomes: emerging gene families and new insights into polydnavirus replication. *Annual Review of Entomology* 49, 431–456.

Kroemer, JA; Webb, BA (2005) IkB-related vankyrin genes in the *Campoletis sonorensis* Ichnovirus: temporal and tissue-specific patterns of expression in parasitized *Heliothis virescens* Lepidopteran Hosts. *Journal of Virology* 79, 7617–7628

Kumar, S; Chandra, A; Pandey, KC (2008) Bacillus thuringiensis (Bt) transgenic crop: An environment friendly insect-pest management strategy. *Journal of Environmental Biology* 29, 641-653

Kwon, B; Kim, Y(2008) Transient expression of an EP1-like gene encoded in *Cotesia plutellae* bracovirus suppresses the hemocyte population in the diamondback moth, *Plutella xylostella*. *Developmental and Comparative Immunology* 32, 932–942

Lawo, NC.; Wackers, FL.; Romeis, J (2009) Indian Bt cotton varieties do not affect the performance of cotton aphids. *PLoS One* 4, e4804

Leluk , J; Schmidt, J; Jones, D (1989) Comparative studies on the protein composition of hymenopteran venom reservoirs. *Toxicon* 27, 105-114.

Lim, U-T; Mahmoud, AMA (2008) Ecotoxicological effect of fenitrothion on *Trissolcus nigripedius* (Hymenoptera: Scelionidae) an egg parasitoid of *Dolycoris baccarum* (Hemiptera: Pentatomidae). *Journal of Asia-Pacific Entomology* 11, 207-210

Liu, Z-C; Ye, G-Y; Hu C; Datta, SK (2003) Impact of transgenic indica rice with a fused gene of cry1Ab/cry1Ac on the rice paddy arthropod community. *Acta Entomologica Sinica* 46, 454-465

Liu, X-X; Zhang, Q-W; Cai, Q-N; Li, J-C; Dong, J (2004) Effect of Bt protein on development of different strains of the cotton bollworm, *Helicoverpa armigera* (Hubner) and the parasitoid, *Microplitis mediator* (Haliday) *Acta Entomologica Sinica* 47, 461-466

Liu, X-x; Zhang, Q-w; Zhao, J-Z; Li, JC; Xu, B-I; Ma, X-m (2005) Effects of Bt transgenic cotton lines on the cotton bollworm parasitoid *Microplitis mediator* in the laboratory. *Biological Control* 35, 134-141

Lövei, GL; S. Arpaia, S (2005) The impact of transgenic plants on natural enemies: a critical review of laboratory studies. *Entomologia Experimentalis et Applicata* 114, 1–14

Lövei, GL, Andow, DA, Arpaia, S (2009) Transgenic insecticidal crops and natural enemies: A detailed review of laboratory studies. *Environmental Entomology* 38(2): 293-306

Lozzia, GC (1999) Biodiversity and structure of ground beetle assemblages (Coleoptera Carabidae) in Bt corn and its effects on nontarget insects. *Bollettino di Zoologia Agraria e di Bachicoltura* 31, 37-58

Manachini, B (2003) Effects of transgenic corn on *Lydella thompsoni* Herting (Diptera Tachinidae) parasitoid of *Ostrinia nubilalis* Hb. (Lepidoptera Crambidae). *Bollettino di Zoologia Agraria e di Bachicoltura* 35, 111-125

Marchetti, E; Alberghini, S; Battisti, A; Squartini, A; Baronio, P; Dindo, ML (2009) Effects of conventional and transgenic *Bacillus thuringiensis* galleriae toxin on *Exorista larvarum* (Diptera: Tachinidae), a parasitoid of forest defoliating Lepidoptera. *Biocontrol Science and Technology* 19, 463-473

Marquini, F; Guedes, RNC; Picanco, MC; Regazzi, AJ (2002) Response of arthropods associated with the canopy of common beans subjected to imidacloprid spraying. *Journal of Applied Entomology* 126, 550-556

Mellet, MA; Schoeman, AS; Broodryk, SW; Hofs, JL (2004) Bollworm (Helicoverpa armigera (Hubner), Lepidoptera: Noctuidae) occurrences in Bt- and non-Bt-cotton fields, Marble Hall, Mpumalanga, South Africa. *African Entomology* 12, 107-115

Mohan, M; Sushil, SN; Bhatt, JC; Gujar, GT; Gupta, HS (2008) Synergistic interaction between sublethal doses of *Bacillus thuringiensis* and *Campoletis chlorideae* in managing *Helicoverpa armigera*. *BioControl* (Dordrecht) 53, 375-386

Moreau, SJM; Guillot, S (2005) Advances and prospects on biosynthesis, structures and functions of venom proteins from parasitic wasps. *Insect Biochemistry and Molecular Biology* 35, 1209-1223

Nakamatsu, Y; Fujii, S; Tanaka, T (2002) Larvae of an endoparasitoid, *Cotesia kariyai* (Hymenoptera: Braconidae), feed on the host fat body directly in the second stadium with the help of teratocytes. *Journal of Insect Physiology* 48, 1041-1052.

Nakamatsu, Y; Tanaka, T (2004) Correlation between concentration of hemolymph nutrients and amount of fat body consumed in lightly and heavily parasitized host (*Pseudaletia separata*). *Journal of Insect Physiology* 50, 135-141.

Naveed, M; Salam, A; Saleem, MA; Rafiq, M; Hamza, A (2010) Toxicity of thiamethoxam and imidacloprid as seed treatments to parasitoids associated to control *Bemisia tabaci*. *Pakistan Journal of Zoology*. 42, 559-565

Nealis, VG; Van Frankenhuyzen, K; Cadogan, BL (1992) Conservation of spruce budworm parasitoids following application of *Bacillus thuringiensis* var. *kurstaki* Berliner. *Canadian Entomologist* 124, 1085-1092

Nga, LT; Kumar, P (2008) Contributions of parasitoids and *Bacillus thuringiensis* to the management of diamondbackmoth in highland crucifer production in Da Lat, Vietnam. *Journal of Asia-Pacific Entomology* 11, 59-64

Oliver, JB; Mannion, CM; Klein, MG; Moyseenko, JJ; Bishop, B (2005) Effect of insecticides on *Tiphia vernalis* (Hymenoptera: Tiphiidae) oviposition and survival of progeny to cocoon stage when parasitizing *Popillia japonica* (Coleoptera : Scarabaeidae) larvae. *Journal of Economic Entomology* 98, 694-703.

Oluwafemi, AR; Rao, Q; Wang, X-Q; Zhang, H-Y (2009) Effect of *Bacillus thuringiensis* on *Habrobracon hebetor* during combined biological control of *Plodia interpunctella*. *Insect Science* 16, 409-416

Orr, DB; Landis, DA (1997) Oviposition of european corn borer (Lepidoptera: Pyralidae) and impact of natural enemy populations in transgenic versus isogenic corn. *Journal of Economic Entomology* 90, 905-909

Ozawa, A; Saito, T; Ota, M (2001) Biological control of the American Serpentine leafminer, Liriomyza trifolii (Burgess), on tomato in greenhouses by parasitoids. II. Evaluation of biological control by Diglyphus isaea (Walker) and Dacnusa sibirica Telenga in commercial greenhouses. Japanese Journal of Applied Entomology and Zoology 45, 61-74

Paine, TD; Hanlon, CC; Byrne, FJ (2011) Potential risks of systemic imidacloprid to parasitoid natural enemies of a cerambycid attacking Eucalyptus. *Biological Control*. 56, 175-178

Parkinson, N; Richards, EH; Conyers, C; Smith, I; Edwards, JP (2002a) Analysis of venom constituents from the parasitoid wasp *Pimpla hypochondriaca* and cloning of a cDNA encoding a venom protein. *Insect Biochemistry and Molecular Biology* 32, 792-735

Parkinson, N; Smith, I; Audsley, N; Edwards, JP (2002b) Purification of pimplin, a paralytic heterodimeric polypeptide from venom of the parasitoid wasp *Pimpla hypochondriaca*, and cloning of the cDNA encoding one of the subunits. *Insect Biochemistry and Molecular Biology* 32, 1769-1773

Parkinson, N; Conyers, C; Smith, I (2002c) A venom protein from the endoparasitoid wasp *Pimpla hypochondriaca* is similar to snake venom reprolysin-type metalloproteases. *Journal of Invertebrate Pathology*. 79, 129-131.

Pennacchio, F; Strand, MR (2006) Evolution of developmental strategies in parasitic hymenoptera. *Annual Review of Entomology* 2006. 51:233–258

Pilcher, CD; Rice, ME; Obrycki, JJ (2005) Impact of transgenic *Bacillus thuringiensis* corn and crop phenology on five nontarget arthropods. *Environmental Entomology*. 34, 1302-1316

Polanczyk, RA; Pratissoli, D; Vianna, UR; Santos Oliveira, RG dos (2006) Interaction between natural enemies: *Trichogramma* and *Bacillus thuringiensis* in pest control. *Acta Scientiarum Agronomy* 28, 233-239

Pons, X; Stary, P (2003) Spring aphid-parasitoid (Hom., Aphididae, Hym., Braconidae) associations and interactions in a Mediterranean arable crop ecosystem, including Bt maize. *Anzeiger fuer Schaedlingskunde* 76, 133-138

Prabhaker, N; Morse, JG; Castle, SJ; Naranjo, SE; Henneberry, TJ; Toscano, NC (2007) Toxicity of seven foliar insecticides to four insect parasitoids attacking citrus and cotton pests. *Journal of Economic Entomology* 100, 1053-1061

Pratissoli, D; Polanczyk, RA; Rodrigues Vianna, U; Santos Andrade, G; Santos Oliveira, RG dos (2006) *Trichogramma pratissolii* Querino & Zucchi (Hymenoptera, Trichogrammatidae) performance in *Anagasta kuehniella* (Zeller) (Lepidoptera, Pyralidae) eggs affected by *Bacillus thuringiensis* Berliner. *Ciencia Rural* 36, 369-377

Preetha, G; Manoharan; Stanley, J; Kuttalam, S. (2010) Impact of chloronitunyl insecticide, imidacloprid on egg, egg-larval and larval parasitoids under laboratory conditions. *Journal of Plant Protection Research* 50, 535-540

Provost, B; Varricchio, P.; Arana, E; Espagne, E; Falabella, P; Huguet, E; Scaleia, RL; Cattolico, L; Poirié, M; Malva, C; Olszewski, JA; Pennacchio, F; Drezen, JM (2004) Bracoviruses contain a large multigene family coding for protein tyrosine phosphatases. *Journal of Virology* 78, 13090–13103

Pruetz, G; Brink, A; Dettner, K (2004) Transgenic insect-resistant corn affects the fourth trophic level: effects of *Bacillus thuringiensis*-corn on the facultative hyperparasitoid *Tetrastichus howardi. Naturwissenschaften* 91, 451-454

Pruetz, G.; Dettner, K (2004) Effect of Bt corn leaf suspension on food consumption by *Chilo partellus* and life history parameters of its parasitoid *Cotesia flavipes* under laboratory conditions. *Entomologia Experimentalis et Applicata* 111, 179-187

Pruijssers, AJ; Strand MR (2007) PTP-H2 and PTP-H3 from *Microplitis demolitor* bracovirus localize to focal adhesions and are antiphagocytic in insect immune cells. *Journal of Virology* 81, 1209–1219

Quicke, DLJ (1997) Physiological interactions of parasitic wasps and their hosts. *in Parasitic Wasps*, Chapman & Hall, London

Ramirez-Romero, R; Bernal, JS.; Chaufaux, J; Kaiser, L (2007) Impact assessment of Bt-maize on a moth parasitoid, *Cotesia marginiventris* (Hymenoptera : Braconidae), via host exposure to purified Cry1Ab protein or Bt-plants. *Crop Protection* 26, 953-962

Raposo, F; Oliveira, L; P. Garcia, P (2003) Susceptibility to insecticides of *Glyptapanteles militaris* (Hymenoptera: Braconidae), a parasitoid of *Pseudaletia unipuncta* (Lepidoptera: Noctuidae). *Biocontrol Science and Technology* 13, 261-26

Reyes, SG; Jovillano-Mostoles, M; Dulce, A (2005) XDiversity, community structure and wet season population abundance of insects in Bt-corn agroecosystem in two sites on Luzon Island, Philippines. *Asia Life Sciences* 14, 55-73

Reyes, SG (2005) Wet season population abundance of *Micraspis discolor* (Fabr.) (Coleoptera:Coccinellidae) and *Trichomma cnaphalocrosis* Uchida (Hymenoptera: Ichnuemonidae) on three transgenic corn hybrids in two sites in the Philippines. *Asia Life Sciences* 14, 217-224

Riquelme V; Maria B; Botto, EN; Lafalce, C (2006) Efficacy of insecticides against the tomato moth *Tuta absoluta* (Lepidoptera: Gelechiidae) and their residual effects on the parasitoid *Trichogrammatoidea bactrae* (Hymenoptera: Trichogrammatidae). *Revista de la Sociedad Entomologica Argentina* 65, 57-65

Rivers, DB; Uckan, F; Ergin, E (2006) Characterization and biochemical analysis of venom from the ectoparasitic wasp Nasonia vitripennis (Walker)(Hymenoptera: Pteromalidae) *Archives of Insect Biochemstry and Physiology* 61, 24-41.(2006)

Rowell, B; Bunsong, N; Satthaporn, K; Phithamma, S; Doungsa-Ard, C (2005) Hymenopteran parasitoids of diamondback moth (Lepidoptera: Ypeunomutidae) in northern Thailand. *Journal of Economic Entomology* 98, 449-456

Sanders, CJ; Pell, JK; Poppy, GM; Raybould, A; Garcia-Alonso, M; Schuler, TH (2007) Host-plant mediated effects of transgenic maize on the insect parasitoid *Campoletis sonorensis* (Hymenoptera : Ichneumonidae). *Biological Control* 40, 362-369

Schlenke, TA; Morales, J; Govind, S; Clark, AG (2007) Contrasting infection strategies in generalist and specialist wasp parasitoids of *Drosoplila melanogaster*. *PLoS Pathogens* 3, 1486-1501.

Schoenly, KG; Cohen, MB; Barrion, AT; Zhang, W; Gaolach, B; Viajante, VD (2003) Interaction of resistant plants and wasp parasitoids of tobacco budworm (Lepidoptera: Noctuidae). *Environmental Biosafety Research* 2, 181-206

Schuler, TH; Poppy GM; Kerry BR; Denholm, I (1999) Potential side effects of insect-resistant transgenic plants on arthropod natural enemies. *Trends in Biotechnology* 12, 210–216.

Schuler, TH; Denholm, I; Jouanin, L; Clark, S. J; Clark, AJ; Poppy, GM (2001) Population-scale laboratory studies of the effect of transgenic plants on nontarget insects. *Molecular Ecology* 10, 1845-1853

Schuler, TH; Potting, RPJ; Denholm, I; Clark, SJ; Clark, AJ; Stewart, CN; Poppy, GM (2003) Tritrophic choice experiments with Bt plants, the diamondback moth (*Plutella xylostella*) and the parasitoid *Cotesia plutellae*. *Transgenic Research* 12, 351-361

Schuler, TH; Denholm, I; Clark, SJ; Stewart, CN; Poppy, GM (2004) Effects of Bt plants on the development and survival of the parasitoid *Cotesia plutellae* (Hymenoptera: Braconidae) in susceptible and Bt-resistant larvae of the diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae). *Journal of Insect Physiology* 50, 435-443

Sehrawat, S; Lal, R; Dahiya, KK (2002) Efficacy of different insecticides and *Trichogramma chilonis* Ishii in managing rice leaffolder, *Cnaphalocrocis medinalis* Guenee. *Pesticide Research Journal* 14, 153-157

Serbielle, C; Chowdhury, S; Pichon, S; Dupas, S; Lesobre, J, Purisima, EO; Drezen, JM; Huguet, E (2008) Viral cystatin evolution and three-dimensional structure modelling: A case of directional selection acting on a viral protein involved in a host-parasitoid interaction. *BMC Biology* 6:38

Sharma, HC; Arora, R; Pampapathy, G (2007) Influence of transgenic cottons with *Bacillus thuringiensis* cry1Ac gene on the natural enemies of *Helicoverpa armigera*. *BioControl* (Dordrecht) 52, 469-489

Sharma, HC; Dhillon, MK; Arora, R (2008) Effects of *Bacillus thuringiensis* delta-endotoxin-fed *Helicoverpa armigera* on the survival and development of the parasitoid *Campoletis chlorideae*. *Entomologia Experimentalis et Applicata* 126, 1-8

Shelby, KS; Webb. BA (1999) Polydnavirus-mediated suppression of insect immunity. *Journal of Insect Physiology* 45, 507-514.

Shi, M; Chen, Y-F; Huang, F; Zhou, X-P; Chen, X-X (2008) Characterization of a novel *Cotesia vestalis* polydnavirus (CvBV) gene containing a ser-rich motif expressed in *Plutella xylostella* larvae. *BMB reports* 587-592

Sisterson, MS; Tabashnik, BE (2005) Simulated effects of transgenic bt crops on specialist parasitoids of target pests. *Environmental Entomology* 34, 733-742

Stelinski, LL; Pelz-Stelinski, KS; Liburd, OE; Gut, LJ (2006) Control strategies for *Rhagoletis mendax* disrupt host-finding and ovipositional capability of its parasitic wasp, *Diachasma alloeum*. *Biological Control*. 36, 91-99

Strand, MR; Ratner, S; Vinson, SB (1983) Maternally induced host regulation by the egg parasitoid *Telenomus heliothidis*, *Physiological Entomology* 8, 469-475.

Strand, MR (1986) The physiological interactions of parasitoids with their hosts and their influence on reproductive strategies. *in Insect Parasitoids*, Academic Press, pp97-136.

Strand, MR; Meola SM; Vinson, SB (1986) Correlating pathological symptons in Heliothis virescens eggs with development of the parasitoid *Telenomus heliothidis*. *Journal of Insect Physiology* 32, 389-402

Strand MR; Witherell, A; Trudeau, D (1997) Two *Microplitis demolitor* polydnavirus mRNAs expressed in hemocytes of *Pseudoplusia includens* contain a common cysteine-rich domain. *Journal of Virology*. 71, 2146–2156

Suderman, RJ; Pruijssers, AJ; Strand, MR (2008) Protein tyrosine phosphatase-H2 from a polydnavirus induces apoptosis of insect cells. *Journal of General Virology* 89, 1411-1420

Takada, Y; Kawamura, S; Tanaka, T (2000) Biological characteristics: growth and development of the egg parasitoid *Trichogramma dendrolimi* (Hymenoptera: Trichogrammatidae) on the cabbage armyworm *Mamestra brassicae* (Lepidoptera: Noctuidae) *Applied Entomology and Zoology* 35, 369-379.

Takada, Y; Kawamura, S; Tanaka, T (2001) Effects of various insecticides on the development of the egg parasitoid *Trichogramma dendrolimi* (Hymenoptera: Trichogrammatidae). *Journal of Economic Entomology* 94, 1340-1343

Takeda, T; Nakamatsu, Y; Tanaka, T (2006) Parasitization by *Cotesia plutellae* enhances detoxifying enzyme activity in *Plutella xylostella*. *Pesticide Biochemistry and Physiology* 86, 15-22

Tanaka, T; Agui, N; Hiruma, K (1987) The parasitoid Apanteles kariyai inhibits pupation of its host, Pseudaletia separata, via disruption of prothoracicotropic hormone release. *General and Comparative Endocrinology* 67, 364-374.

Tanaka, T; Nakamatsu, Y; Harvey, JA (2006) Strategies during larval development of hymenopteran parasitoids in ensuring a suitable food resource. *Proceeding of Arthropodan Embryological Society of Japan*. 41, 11-19

Thompson, SN; Redak, RA; Wang, LW (2001) Altered dietary nutrient intake maintains metabolic homeostasis in parasitized larvae of the insect *Manduca sexta* L. *The Journal of Experimental Biology* 204, 4065–4080.

Thompson, SN; Redak, RA; Borchardt, DB (2002) The glucogenetic response of a parasitized insect *Manduca sexta* L. is partially mediated by differential nutrient intake. *Biochimica et Biophysica Acta* 1571, 138-150(2002)

Tounou, A-K; Gounou, S; Borgemeister, C; Goumedzoe, YMD.; Schulthess, F (2005) Susceptibility of *Eldana saccharina* (Lepidoptera : Pyralidae), *Busseola fusca* and *Sesamia calamistis* (Lepidoptera : Noctuidae) to *Bacillus thuringiensis* Cry toxins and potential side effects on the larval parasitoid *Cotesia sesamiae* (Hymenoptera : Braconidae). *Biocontrol Science and Technology*. 15, 127-137

Ulpah, S; Kok, LT (1996) Interrelationship of *Bacillus thuringiensis* Berliner to the diamondback moth (Lepidoptera: Noctuidae) and its primary parasitoid, *Diadegma insulare* (Hymenoptera: Ichneumonidae). *Journal of Entomological Science* 31, 371-377

Varlez, S; Jervis, MA; Kidd, NAC; Campos, M; McEwen, PK (1993) Effects of *Bacillus thuringiensis* on parasitoids of the olive moth, *Prays oleae* Bern. (Lep., Yponomeutidae). *Journal of Applied Entomology* 116, 267-272

Vojtech, E; Meissle, M; Poppy, GM (2005) Effects of Bt maize on the herbivore *Spodoptera littoralis* (Lepidoptera : Noctuidae) and the parasitoid *Cotesta marginiventris* (Hymenoptera : Braconidae). *Transgenic Research* 14, 133-144

Walker, GP; Cameron, PJ; MacDonald, FM; Madhusudhan, VV; Wallace, AR (2007) Impacts of *Bacillus thuringiensis* toxins on parasitoids (Hymenoptera : Braconidae) of *Spodoptera litura* and *Helicoverpa armigera* (Lepidoptera : Noctuidae). *Biological Control* 40, 142-151

Wang X; Liu S (1998) Bionomics of *Diadromus collaris* (Hymenoptera: Ichneumomonidae), a major pupal parasitoid of *Plutella xylostella. Acta Entomologica Sinica* 41, 389-395

Wang DS; Lv LH; He YR; Qin SS; Pan F (2010) Effect of conventional insecticides on *Trichogrammatoidea bactrae*. *Chinese Bulletin of Entomology* 47, 379-383

Wang, X. & Liu, S. (1998) Bionomics of *Diadromus collaris* (Hymenoptera, Ichneumononidae), a major pupal parasitoid of *Plutella xylostella*. *Acta Entomologica Sinica* 41, 389-395

Weaver, RJ; Marris, GC; Olieff, S; Moson, JH; Edwards, JP (1997) Role of ectoparsitoid venom in the regulation of haemolymph ecdysteroid titers in a host noctuid moth. *Archives of Insect Biochemistry and Physiology* 35, 169-178

Webb, BA; Strand, MR (2005) The biology and genomics of polydnaviruses. *in Comprehensive Molecular Insect Science* 6, eds. Gilberet, LI, Iatrou, K, Gill, SS, Elsevier, San Diego pp. 323-360

Wei, W.; Schuler, TH; Clark, SJ; Stewart, CN, Jr.; Poppy, GM (2008) Movement of transgenic plant-expressed Bt Cry1Ac proteins through high trophic levels. *Journal of Applied Entomology* 132, 1-11

Whitehouse, MEA; Wilson, L. J.; Constable, GA (2007) Target and non-target effects on the invertebrate community of Vip cotton, a new insecticidal transgenic. *Australian Journal of Agricultural Research* 58, 383

Whitfield, JB (2002) Estimating the age of the polydnavirus/braconid wasp symbiosis. *Proceedings of the National Academy of Sciences of the United States of America*, 99, 7508-7513

Whitfield, JB; Asgari, S (2003) Virus or not? Phylogenetics of polydnaviruses and their wasp carriers. *Journal of Insect Physiology* 49, 397-405

Wise, JC; Jenkins, PE; Poppen, RV; Isaacs, R (2010) Activity of broad-spectrum and reduced-risk insecticides on various life stages of cranberry Fruitworm (Lepidoptera: Pyralidae) in highbush blueberry. *Journal of Economic Entomology* 103, 1720-1728

Wolfenbarger, LL; Naranjo, SE; Lundgren, JG; Bitzer, RJ; Watrud, LS (2008) Bt crop effects on functional guilds of non-target arthropods: A meta-analysis. *PLoS One 3*
Xu, Y-Y; Liu, T-X; Leibee, GL; Jones, WA (2004) Effects of selected insecticides on *Diadegma insulare* (Hymenoptera: Ichneumonidae), a parasitoid of *Plutella xylostella* (Lepidoptera: Plutellida). *Biocontrol Science and Technology* 14, 713-723

Xu, Y-Y; Liu, T-X; Leibee, GL; Jones, WA (2004) Effects of selected insecticides on *Diadegma insulare* (Hymenoptera: Ichneumonidae), a parasitoid of *Plutella xylostella* (Lepidoptera: Plutellida). *Biocontrol Science and Technology* 14, 713-723

Zhang, N; Ren, Q; Liu, XX; Zhao, ZW; Zhang, QW (2010) Behavioral responses of female *Microplitis tuberculifer* to volatiles from cotton plants induced by herbivores (Hymenoptera: Braconidae). *Entomologia Generalis* 32, 201-210

Part 2

Health Risks Associated to Insecticides

6

Health and Insecticides

Nadeem Sheikh
University of the Punjab, Quaid-i-Azam Campus, Lahore, Pakistan

1. Introduction

Pyrethroids are synthetic chemicals similar to pyrethrins in the pyrethrum extract which is obtained from Chrysanthemum plant. Historically, pyrethroids have been classified into two classes that differ in their chemical structure and symptoms of exposure:
Type I pyrethroids include allethrin, tetramethrin, d-phenothrin, permethrin, and bioresmethrin.
Type II pyrethroids include cypermethrin, cyphenothrin, deltamethrin, cyfluthrin, fenvalerate , (Klaassen *et al*, 1996; Ray, 1991).
Pyrethroid kills the insects that eat or come in contact with it by quickly affecting the insect's central nervous system (Tomlin, 1994; Costa, 1997).
Cypermethrin is one of the most widely used type II pyrethroid insecticide, first synthesized in 1974 (WHO, 1989; Patel *et al*, 2006).

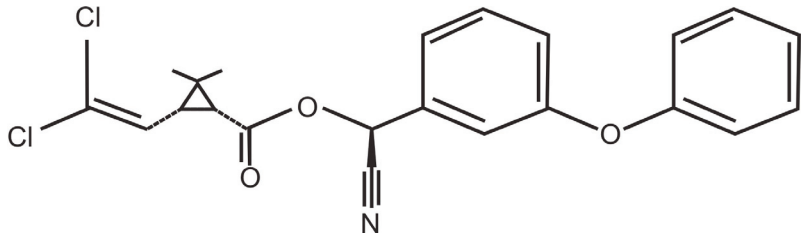


Fig. 1. Structural Formula of Cypermethrin; Molecular formula: $C_{22}H_{19}Cl_2NO_3$; Molecular weight: 416.3

It is considered to be environmentally safe and widely used in agriculture and veterinary medicine (Shukla *et al*, 2002). It is classified by the World Health Organization (WHO) as "moderately hazardous" *(WHO Recommended Classification of Pesticides by Hazard 1994-95, WHO, Geneva)*. It is a fast-acting neurotoxin and is known to cause free radical-mediated tissue damage (Patel *et al*, 2006). Like other pyrethroids, cypermethrin kills the insects by interacting with the sodium channels in nerve cells through which sodium enters the cell in order to transmit a nerve signal. These channels can remain open for up to seconds instead of the normal period of a few milliseconds, after a signal has been transmitted. Cypermethrin also interferes with other receptors in the nervous system. The effect is that of long-lasting trains of repetitive impulses in sense organs. (Rodriguez *et al,* 2008; Tomlin, 1994; Costa, 1997).

Cypermethrin may become an air pollutant and its toxic effects in humans include abnormal facial sensations, coughing, dizziness, tingling, burning, itching, headache, nausea, anorexia and fatigue, vomiting and increased stomach secretion. It is also a skin and eye irritant (Klaassen *et al*, 1996; Sitting, 1991). Patients with severe exposure to cypermethrin may suffer from muscular twitching, coma and convulsive attacks. Mice on exposure to cypermethrin display symptoms including writhing, convolutions, salivation, etc (Lawrence *et al*, 1982). Chronic symptoms after exposure to cypermethrin include brain and locomotory disorders, polyneuropathy and immuno-suppression, and resemble the multiple chemical sensitivity syndromes (Müller-Mohnssen, 1995).

Cypermethrin is classified by the US EPA as a weak category C oncogen, a possible human carcinogen with evidences of carcinogenesis in animals but no evidence of carcinogenicity in humans (US EPA, 1989; US EPA, 1997). It possesses complete carcinogenic and co-carcinogenic potential (tumor initiating and promoting potential) in both the sexes of Swiss albino mice and male as well as female mice develop benign tumors in skin upon exposure to cypermethrin (Shukla *et al*, 2002).

Cypermethrin is genotoxic in mouse spleen and bone marrow cells where it induces the chromosomal aberration and sister chromatid exchange (Amer *et al*, 1993; Giri *et al*, 2003). It induces systemic genotoxicity in mammals by causing DNA damage in vital organs like brain, liver, kidney, apart from that in the hematopoietic system (Patel *et al*, 2006). It possesses mutagenic activity inducing dominant lethal mutations in male germ cells of mice (Shukla *et al*, 2002). It induces chromosomal aberrations and single stranded breaks in DNA in the cultured human lymphocytes. Moreover, it also affects the cell cycle causing a decrease in the proliferative rate index (Puig *et al*, 1989).

Lungs are an important entry point for airborne contaminants (e.g., toxic gases, particulates, aerosols, volatile organic solvents etc). Toxicants present in the breathing zone may be absorbed in the nasopharyngeal, tracheobronchial, or pulmonary exchange surfaces of the lung, depending upon the physical and chemical properties of the toxicant. But the lung alveoli and the terminal bronchioles are one of the most effective surfaces in the body for absorption and are responsible for most of the resultant toxicity that occurs during respiratory exposure. Materials that remain within the respiratory system may produce local toxicity that may take form of lung cancer (Richards, 2008).

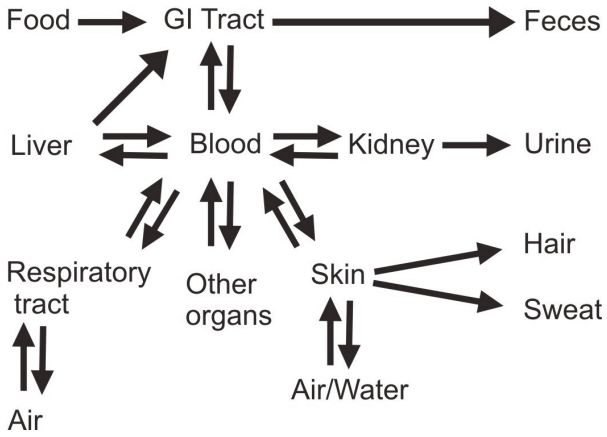


Fig. 2. Absorption & distribution of chemicals in animals (Apostoli, 2002).

Toxicants may also pass into the systemic circulation from the lungs and affect the other body parts especially the liver because the hepatocytes directly receive the chemicals from the blood (Richards, 2008).

The process of oncogenesis is a progression of events that lead to the formation of a tumor. Of the known carcinogenic agents (viruses, ultraviolet and ionizing radiations, and chemicals), chemicals appear to be of major importance in the induction of human cancers (Richards, 2008; Miller & Miller, 1981). Chemical carcinogenesis is a multistage process which involves initiation, promotion & progression (Simons, 1995; Pitot, 2001; Luch, 2005; Richards, 2008; Miller & Miller, 1981; Lyman, 1992). Initiation requires an irreversible change in the cellular genome within the portions of genome that are involved in regulating process of cellular growth and differentiation, whereas promotion moves initiated cells further along their transformation, commonly associated with prolonged and reversible exposure. Tumor progression results in genotypic and phenotypic changes associated with tumor growth, invasion, and metastasis (Richards, 2008; Miller & Miller, 1981; Lyman, 1992).

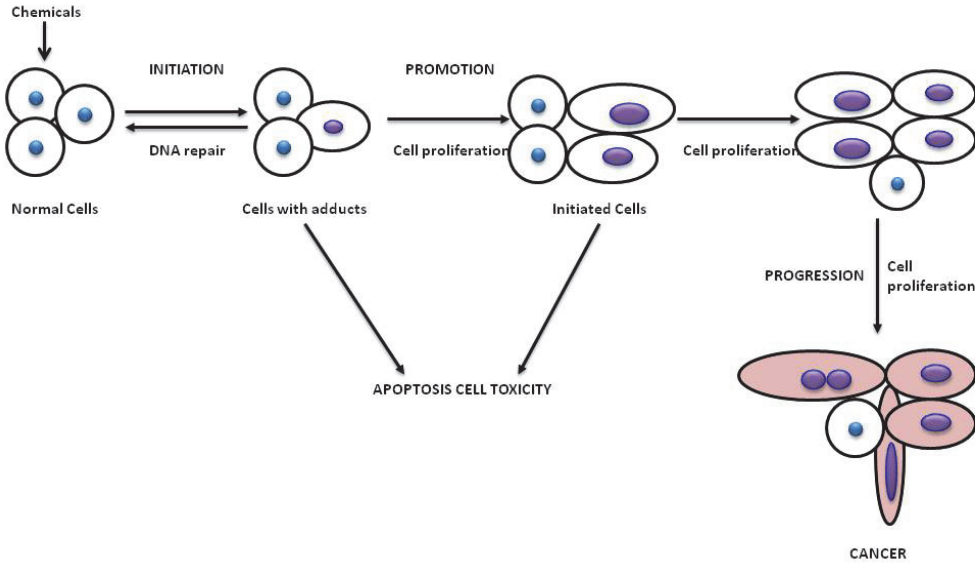


Fig. 3. Mechanism of the Chemical Carcinogenesis (Adopted from Oliveira *et al*, 2007).

The process of oncogenesis requires several changes in the normal properties of a cell. Typically the malignant cell properties include acquisition of self-sufficiency in growth signals and loss of sensitivity to anti-growth signals (leading to uncontrolled growth), loss of capacity for apoptosis, acquisition of sustained angiogenesis, acquisition of ability to invade neighbouring tissues, acquisition of ability to build metastasis at distant sites and loss of capacity to repair genetic errors (Hanahan and Weinberg, 2000).

Lung cancer is a disease of uncontrolled cell growth in tissues of the lung. This growth may lead to metastasis, invasion of adjacent tissue and infiltration beyond the lungs. The vast majority of primary lung cancers are carcinomas of the lung, derived from epithelial cells (Ashley, 1980). The major causes of lung cancer are chemical carcinogens such as those in

tobacco smoke and air pollutants, ionizing radiation as radon gas, and viral infection (Lombard, 2006; Kotin & Falk,1959; Stock & Campbell,1955).

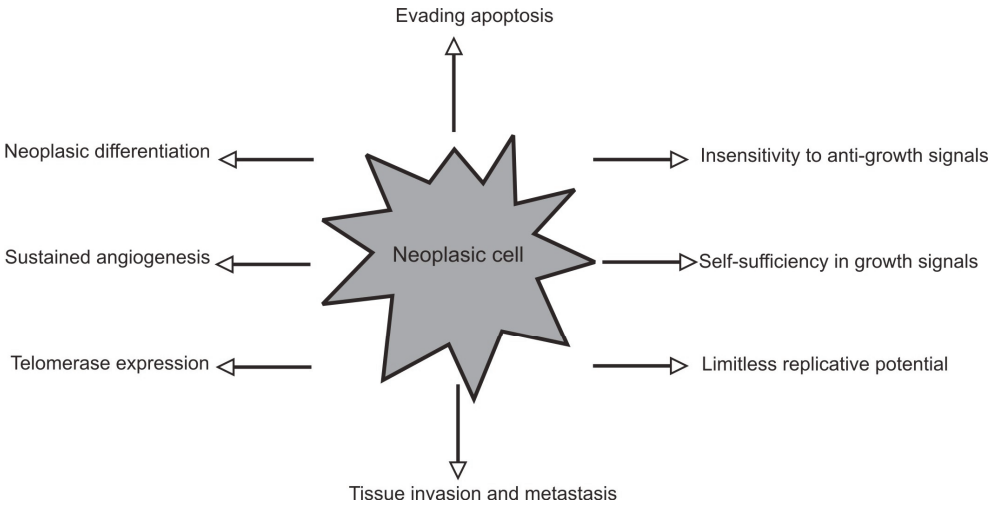


Fig. 4. Malignant cell characteristics (Oliveira *et al*, 2007).

There are many histopathologic types of lung cancer and World Health Organization (WHO) classification, recommended originally in 1977 is usually accepted as the definitive classification of this cancer. Major types of lung cancer are adenocarcinoma, squamous cell carcinoma and large cell carcinoma collectively known as non-small-cell lung cancer (NSCLC) accounting for 75-80% of all lung cancers and small-cell lung cancer (SCLC) comprises the remaining 20-25% of lung cancer cases (Colby *et al*, 1995).

Adenocarcinoma is the most common form of lung cancer originating in the peripheral glandular tissues of lung (Guillan *et al*, 1967). These tumors predominantly consists of mucin-secreting or non-mucinous columnar cells arranged as regular or irregular tubular and papillary elements supported by a well or poorly developed fibrous stroma (Ashley & Davies, 1967).

Squamous cell carcinoma also called epidermoid carcinoma usually appears as central tumors consisting mainly of clearly defined flattened or polygonal cells of pavement type arranged in keratinizing whorls, irregular masses and narrow or wide anatomizing or branching columns dispersed haphazardly through a fibrous stroma (Smith & Dexter, 1963).

Large cell or bronchiolar carcinoma is the least common of all NSCLCs. It is composed of large cells with prominent nucleoli, and no mucin production or intercellular bridging is identified. A variant of large cell carcinoma has been identified; it contains neuroendocrine features and is called large cell neuroendocrine carcinoma (Zhiyong *et al*, 2006).

Small cell lung carcinomas (SCLC) typically are centrally located, arising in peribronchial locations. These are richly cellular neoplasms consisting of more or less regular, darkly staining cells with characteristic nuclei, which because of their shape have been linked to oat grains. These nuclei are set in a cytoplasm so flimsy that it is barely discernible and common

appearance is that of nuclei closely knit and lying in an indistinct web- like cytoplasm with cell membrane rarely clearly defined (Chaudhuri, 1973; Wurschmidt *et al*, 1996).

Cancer of the liver refers to the uncontrolled and abnormal growth of cells in the liver. Most cases of the liver carcinomas are not primary but are the result from the metastases of other tumors as that of GI tract and lung cancer. The most frequent, malignant, primary liver cancer is hepatocellular carcinoma (HCC) an aggressive hepatic neoplasm which usually occurs in combination with cirrhosis (Gall, 1960). It has been suggested that HCC arises as a very well differentiated cancer and proliferates with a stepwise process of dedifferentiation of mature cells. Many HCCS seem to arise from dysplastic nodules (DNs) on the basis of evidence of the DNs containing HCC foci, frequent association of DNs in the vicinity of HCC, and clinical progression from DN to HCC (Kojiro and Roskams, 2005).

Microscopically, hepatocellular carcinoma has four cytological and architectural patterns: fibrolamellar, pseudoglandular (adenoid), pleomorphic (giant cells) and clear cells. In well differentiated forms, tumor cells resemble hepatocytes, for trabeculae, cords and nests, and may contain bile pigment in cytoplasm. In poorly differentiated forms, malignant epithelial cells are discohesive, pleomorphic, anaplastic and giant. The tumor has a scant stroma and central necrosis because of the poor vascularization (Hou & McFadzean, 1956; Takayasu *et al*, 2004).

Cholangiocarcinoma constitutes 10-22% of all the primary epithelial cancer of the liver and occurs most often in non-cirrhotic tissues (Menias et al, 2008; Patton & Horn, 1964; Steiner, 1957).It is a malignancy of biliary duct system that may originate in the liver and extrahepatic bile ducts, which terminate at the ampulla of Vater (Lake, 1993). A carcinoma arising from the intrahepatic bile ducts is typically a well differentiated adenocarcinoma that exhibit glandular or acinar structures; intracytoplasmic mucin is almost always observed. Characteristically, cells are cuboidal or low columnar and resemble biliary epithelium. A dense fibrous stroma is characteristic and may dominate the histologic architecture. It tends to invade lymphatics, blood vessels, perineural and periductal spaces, and portal tracts. A dense fibrous stroma is characteristic and may dominate the histologic architecture. It tends to invade lymphatics, blood vessels, perineural and periductal spaces, and portal tracts (Ashley, 1980).

Cypermethrin and other pyrethroids have been used worldwide in the fields, gardens and homes for the last few decades. Although these insecticides have benefits but they also have side effects especially when they are used in increasing amount. Continuous exposure to these insecticides may cause severe disorders especially the mutations in the genome leading to cancer.

The histopathological changes in the lung and liver tissues of cypermethrin exposed mice have shown significant changes. Exposure of the mice to cypermethrin through inhalation induces significant time dependent changes in the histopathology of lung as well as liver tissue. Inhalation is a major route of exposure to air born pollutants such as the pesticides (Emmendoerffer *et al*., 2000; Richards, 2008). Lungs and liver are the organs which are at highest risk to the environmental pollutants especially the air born chemicals (Dixon *et al*., 2008). Cypermethrin has shown the carcinogenic effect in the lung tissues of mice. Exposure to cypermethrin caused a gradual distortion of normal structure of alveoli in the lung. Cypermethrin induces hyperplasia and necrosis among the alveolar cells. There was also inflammation of lung tissue leading to pulmonary edema and alveolitis. It also induced pulmonary fibrosis due to which size of alveolar sac was reduced and alveolar walls became thicker.

Epidemiologic data showed an increase in the number of cancer cases in persons involved in agricultural production using pesticides (Kornuta *et al*., 1996). Toxicological studies suggest

that cypermethrin and other pyrethroids have carcinogenic effects (Shukla *et al*., 2002). There is evidence that pyrethroids induce benign tumors in the lungs of mice (Ishmael and Lithfield, 1988). Inhalation of pyrethroids may cause alveolitis, pulmonary edema, and damage to lung cells (Tian, 1993). Lung cancer develops through a series of progressive pathological changes occurring in the respiratory epithelium (Vineis and Husgafvel-Pursiainen, 2005)

Pyrethroids are known to be genotoxic and may interact with DNA and damage its structure. These induce chromosomal aberrations and single strand breaks in DNA (Kornuta *et al*., 1996; Puig *et al*., 1989). Pyrethroids may lead to the molecular alterations such as loss of heterozygosity; gene mutations and aberrant gene promoter methylation which are potentially promising molecular biomarkers of lung carcinogenesis (Vineis and Husgafvel-Pursiainen, 2005).

It has been suggested that pyrethroids induce the oxidative stress in lungs (Kale *et al*., 1999a). Oxidative stress is caused by an imbalance in the production of reactive oxygen. A particularly destructive aspect of oxidative stress is the production of reactive oxygen species, which includes free radicals and peroxides which cause damage to the cells (Schafer and Buettner, 2001; Rahman and MacNee, 2001; Evans and Cooke, 2004). DNA damage is caused by oxygen-derived species as free radicals resulting from oxidative stress (Abdollahi *et al*., 2004). Oxidative stress may cause pathological changes in the pulmonary epithelium. It may lead to the inflammation of lung tissue. There may be hyperplasia or proliferation of alveolar cells (MacNee, 2001; Erdogan *et al*., 2006). Intense stresses may cause necrosis of the alveolar cells (Lennon *et al*., 1991).

Toxicological studies suggest that pyrethroids may induce inflammation of the lung tissues causing the oxidative stress (Emmendoerffer *et al*., 2000) and collagen deposition leading to the pulmonary fibrosis and edema (Erdogan *et al*., 2006). Idiopathic pulmonary fibrosis (IPF) is found to be associated with lung cancer as the epidemiological studies show greater incidences of lung cancer among the patients having IPF (Park *et al*., 2001). cypermethrin has also been seen to induce the liver injury. Exposure to cypermethrin damages the normal architecture of liver lobules. The number of the hepatocytes was found to be reduced with distorted polygonal shapes and widened sinusoids. Cypermethrin also induces liver fibrosis and necrosis. The magnitude of these findings was time dependent being more prominent in the tissues exposed for greater time (unpublished data).

Pyrethroids are known to have hepatotoxic potential in mammals as they cause histopathological damage of liver through inhalation exposure (Tuzmen *et al*., 2008; Mani *et al*., 2004; Okuno *et al*., 1986; Sakr, 1999). Cypermethrin induces distortion of the normal polygonal shape of the hepatocytes as they become irregular and exhibit cloudy swelling. Histopathological changes induced by the cypermethrin include the liver vacuolar degeneration, enlargement of the sinusoids between hypertrophied hepatocytes, degeneration in hepatic cords and hepatocytes, vacuole formations in hepatocytes, pleomorphism in nucleus, congestion (Yavasoglu *et al*., 2006; Ksheerasagar and Kaliwal, 2006), extensive vacuolated pycnosis and necrosis (Singh and Singh, 2008; Abou-Zaid and El-Balshy, 1995; Sakr and Hanafy, 2002). Evidences have shown that hepatotoxic chemicals as cypermethrin may induce liver fibrosis (Singh and Singh, 2008). As in the case of lungs, liver damage and fibrosis is also induced by oxidative stress (Abdollahi *et al*., 2004). Exposure to cypermethrin introduces significant oxidative stress in the hepatic tissues due to which release of free radicals occurs in the liver. These free radicals cause destruction of the normal hepatic tissues (Giray *et al*., 2001; Kale *et al*., 1999b).

The inhalation exposure of mice to cypermethrin resulted in the development of skin tumor epidermal in origin as reported that cypermethrin and other pyrethroids possess carcinogenic (tumor initiating) and cocarcinogenic (tumor promoting) potential (Shukla *et al*., 2002; Kornuta *et al*., 1996). The carcinogenic property of cypermethrin observed may be attributed to its ability to interact with DNA and damage its structure (Kornuta *et al*., 1996). Such interactions are critical for the initiation of cells to transform into neoplastic cells. Cypermethrin may also induce the frequency of well established markers of genotoxicity such as chromosomal aberrations and micronuclei formation (Suralles *et al*., 1995). Commercial formulations of cypermethrin are reported to cause *in vivo* induction of sister chromatid exchange in mouse bone marrow cells (Chauhan *et al*., 1997). Taken together these reports we can conclude that cypermethrin causes hazardous effects in non target organisms through inhalation exposure. It has potential to induce carcinogenesis and fibrosis of the lungs and liver. Further more severe exposure of cypermethrin may cause the development of skin tumor due to mutations in the genome. However, further studies on carcinogenicity and acute as well as chronic toxicity of cypermethrin and other pyrethroids are required. In the agriculture sector of Pakistan about 70% of the population lives in villages and most of them are involved in agriculture directly or indirectly. The farmers use pyrethroids to save their crops from pest attacks. The tendency of farmers to use pyrethroids is increasing which is alarming. Also there are many malpractices, i.e., the spray men do not follow the necessary precautions. So it is strongly recommended that these workers should be educated about the harmful and toxic effects of the synthetic pesticides. Moreover, they should know the importance of the protective measures during spraying. Rural workers and public health authorities must become aware of the importance of protective equipment, periodic health examinations and reduced environmental pollution in order to lessen occupational risk of field workers and promote improved conditions of life for the population at large scale.

2. References

Abdollahi, M., Ranjbar, A., Shadnia, S., Nikfa, S. And Rezaei, A., 2004. Pesticides and oxidative stress: a review. *Med. Sci. Monit.,* 10(6): 141-147.

Abou-Zaid, F.A. And El-Balshy, R.M., 1995. Toxic effect of Ezalo, a synthetic pyrethroid on liver, kidney and lung of newly born mice. *J. Union Arab Biol.,* 4: 235-264.

Amer, S.M., Ibrahim, A.A.E.S., El-Sherbeny, K.M., 1993. Induction of chromosomal aberrations and sister chromatid exchange *in vivo* and *in vitro* by the insecticide cypermethrin. *J. Appl. Toxicol.,* 13(5): 341-345.

Apostoli, P., 2002. Element in environmental and occupational medicine. *J. Chromatogr. B*., 778: 63-97.

Ashley, 1980. *Evan's Histological Appearances of Tumours.* 3rd Ed. Churchill Livingstone Inc., New York. pp. 633-653.

Ashley, D.J.B. And Davies, H.D., 1967. Cancer of the lung. Histology and biological behavior. *Cancer,* 20(2): 165-174.

Chaudhuri, M.R., 1973. Primary pulmonary cavitating carcinomas. *Thorax*, 28(3): 354-366.

Chauhan, L.K., Agarwal, D.K. And Sundararaman V., 1997. In vivo induction of sister chromatid exchange in mouse bone marrow following oral exposure to commercial formulations of alpha cyano pyrethroids. *Toxicol. Lett.,* 93:153–157.

Colby, T.V., Koss, M.N. And Travis, W.D. (Eds), 1995. *Atlas of Tumor Pathology. Tumors of the Lower Respiratory Tract.* Armed Forces Institute of Pathology, Washington, DC.

Costa, L.G., 1997. Basic Toxicology of Pesticides. In: Human Health Effects of Pesticides. Occupational Medicine. State of the Art Reviews. Keifer, M. C. (Ed.), (Vol.12 No.2). Hanley and Belfus. Inc., Philadelphia. pp. 251-268.

Dixon, D., Herbert, R.A., Kissling, G.E., Brix, A.E., Miller, R.A. And Maronpot, R.R., 2008. Summary of chemically induced pulmonary lesions in the National Toxicology Program (NTP) toxicology and carcinogenesis studies. *Toxicol.Pathol.,* 36(3): 428-439.

Erdogan, S., Zeren, E.H., Emre, M., Aydin, O. And Gumurdulu, D., 2006. Pulmonary effects of deltamethrin inhalation: an experimental study in rats. *Ecotoxicol. Environ. Safety,* 63(2): 318-323.

Evans, M.D. And Cooke, M.S., 2004. Factors contributing to the outcome of oxidative damage to nucleic acids. *BioEssays*., 26(5): 533-542.

Gall E. A., Primary and metastatic carcinoma of the liver. Relationship to hepatic cirrhosis. *Arch Pathol*.1960Aug;70:226-32.

Giray, B., Gürbay, A. And Hincal, F., 2001. Cypermethrin-induced oxidative stress in rat brain and liver is prevented by Vitamin E or allopurinol. *Toxicol. Lett.,* 118(3): 139-146.

Giri, S., Giri, A., Sharma, G.D. And Prasad, S.B., 2003. Induction of sister chromatid exchanges by cypermethrin and carbosulfan in bone marrow cells of mice *in vivo*. *Mutagenesis,* 18(1): 53-58.

Guillan, R.A., Zelman, S. And Alonso, R.A., 1967. Adenocarcinoma of the lungs. An analysis of 24 cases in men. *Am. J. Clin. Pathol.,* 47(5): 580-584.

Hanahan, D. And Weinberg, R.A., 2000. The hallmarks of cancer. *Cell.,* 100: 57-70.

Hou, P. C., and McFadzean, A. J. S., J. Path. Bact., 1956, 72, 411

Ishmael, J. And Lithfield, M.H. 1988. Chronic toxicity and carcinogenic evaluation of permethrin in rats and mice. *Fundam. Appl. Toxicol.,* 11(2):308-322.

Kale, M., Rathore, N., John, S. And Bhatnagar, D., 1999a. Lipid Peroxidation and Antioxidant Enzymes in Rat Tissues in Pyrethroid Toxicity: Possible Involvement of Reactive Oxygen Species. . *J. Nutr. Environ. Med.,* 9: 37-46.

Kale, M., Rathore, N., John, S., Bhatnagar, D., Nayyar, S.S. And Kothari, V., 1999b. The protective effect of vitamin E in pyrethroid-induced oxidative stress in rat tissues. *J. Nutr. Environ. Med.,* 9(4): 281-287.

Klaassen, C.D., Amdur, M.O., And Doull, J. (Eds.)., 1996. *Casarett and Doull's Toxicology. The Basic Science of Poisons*. 5th Ed. McGraw-Hill Companies, Inc. Toronto.

Kojiro M and Roskams T. Early hepatocellular carcinoma and dysplastic nodules. Semin Liver Dis.2005;25(2):133-42.

Kornuta, N., Bagley, E. And Nedopitanskaya, N., 1996. Genotoxic effects of pesticides. *J. Environ. Pathol. Toxicol. Oncol.,* 15(2-4): 75-78.

Kotin, P., And Falk, H.L., 1959. The role and action of environmental agents in the pathogenesis of lung cancer. I. Air pollution. *Cancer,* 12: 147-163.

Ksheerasagar, R.L. And Kaliwal, B.B., 2006. Histological and biochemical changes in the liver of albino mice on exposure to insecticide, carbosulfan. *Caspian J. Env. Sci.,* 4(1): 67-70.

Lake J.R.Changing indications for liver transplantation. *Gastroenterol Clin North Am*.1993 Jun;22(2):213-29

Lawrence, L.J. And Casida, J.E., 1982. Pyrethroid toxicology.mouse intracerebral structure toxicity relationships. *Pestic. Biochem. Physiol.,* 18(1): 9-14.

Lennon, S.V., Martin, S.J. And Cotter, T.G., 1991. Dose-dependent induction of apoptosis in human tumour cell lines by widely diverging stimuli. *Cell. Prolif.,* 24(2): 203–214.

Lombard, H.L., 2006. An epidemiological study in lung cancer. *Cancer*, 18(10): 1301-1309.

Luch, A., 2005. Nature and nurture-lessons from chemical carcinogenesis. *Nat. Rev. Cancer*, 5(2): 113-125.

Lyman, G.H., 1992. Risk factors for cancer. *Prim. Care,* 19(3): 465-79.
Macnee, W., 2001. Oxidative stress and lung inflammation in airways disease. *Eur. J. Pharmacol.,* 429(1-3): 195-207.
Mani, U., Prasad, A.K., Sureshkumar, V., Kumar, P., Lal, K., Maji, B.K. And Dutta, K.K., 2004. Hepatotoxic alterations induced by subchronic exposure of rats to formulated fenvalerate (20% EC) by nose only inhalation. *Biomed. Environ.Sci.,* 17(3): 309-314.
Menias CO, Surabhi VR, Prasad SR, Wang HL, Narra VR, Chintapalli KN. Mimics of cholangiocarcinoma: spectrum of disease. *Radiographics*. 2008 Jul-Aug;28(4):1115-29
Miller, E.C., Miller, J.A., 1981. Mechanisms of chemical carcinogenesis. *Cancer,* 47(5) Suppl): 1055-1064.
Müller-Mohnssen, H., 1999. Chronic sequelae and irreversible injuries following acute pyrethroid intoxication. *Toxicol. Lett.,* 107(1-3): 161-176.
Okuno, Y., Ito, S., Seki, T., Hiromori, T., Murakami, A.L., Kadota, T. And Miyamoto, I., 1986. Fenvalerate induced granulomatous changes in rats and mice. *J. Toxicol. Sci.,* 11:53-66.
Oliveira Paula A., Colaço Aura, Chaves Raquel, Guedes-Pinto Henrique, De-La-Cruz P. Luis F., Lopes Carlos. Chemical carcinogenesis. An. Acad. Bras. Ciênc.79(4): 593-616.
Park, J., Kim, D.S., Shim, T.S., Lim, C.M., Koh, Y., Lee, S.D., Kim, W.S., Kim, W.D., Lee, J.S. And Song, K.S., 2001. Lung cancer in patients with idiopathic pulmonary fibrosis. *Eur. Respir. J.,* 17: 1216–1219.
Patel, S., Pandey, A.K., Bajpayee, M., Parmar, D. And Dhawan, A., 2006. Cypermethrin-induced DNA damage in organs and tissues of the mouse: Evidence from the comet assay. *Mutat. Res.–Genetic Toxicol. Environ. Mutagen.,* 607(2): 176- 183.
Patton R. B,Horn. R. C. Jr. Primary Liver Carcinoma. Autopsy Study Of 60 Cases. *Cancer*.1964 Jun;17:757-68.
Pitot, H.C., 2001. Animal models of neoplastic development. *Dev. Biol. (Basel),* 106: 53-57.
Puig, M., Carbonell, E., Xamena, N., Creus, A. And Marcos, R., 1989. Analysis of cytogenetic damage induced in cultured human lymphocytes by the pyrethroid insecticides cypermethrin and fenvalerate. *Mutagenesis,* 4(1): 72-74.
Rahman, I. And Macnee, W., 2001. Oxidative stress and regulation of glutathione in lung inflammation. *Eur. Respir. J.,* 16(3): 534-554.
Ray, D.E., 1991. Pesticides derived from plants and other organisms. In: *Handbook of Pesticide Toxicology*. Hayes, Jr. W. J. and Laws, E. R. (Eds.), Vol. 2. Academic Press, Toronto. pp. 585-593.
Richards, I.S., 2008. *Principles and Practices of Toxicology in Public Health*. 1st Ed. Jones and Bartlett Publishers, USA. pp.115-190.
Rodriguez, H., Tamayo, C., Inostroza, J., Soto, C., Bustos-Obregón, E. and Paniagua, R., 2008. Cypermethrin effects on the adult mice seminal glands. *Ecotoxicol. Environ. Saf.,* [Epub ahead of print].
Sakr, S.A. And Hanafy, S.M., 2002. Histological alteration in the liver and kidney of toads (*Bufo regularis*) intoxicated with a pyrethroid insecticide. *OnLine Journal of Biological Sciences,* 2(4): 208-211.
Sakr, S.A., 1999. Pyrethroid inhalation induced hepatotoxicity in albino rats. *Oxford Res. Forum J.,* 1: 27-31.
Schafer, F.Q. And Buettner, G.R., 2001. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic. Biol. Med.,* 30(11): 1191–1212.
Shukla, Y., Yadav, A. And Arora A., 2002. Carcinogenic and cocarcinogenic potential of cypermethrin on mouse skin. *Cancer Lett.,* 182(1): 33-41.

Simons, J.W., 1995. Genetic, epigenetic, dysgenetic, and non-genetic mechanisms in tumorigenesis. *Crit. Rev. Oncog*., 6: 261-273.

Singh, P.B. And Singh, V., 2008. Cypermethrin induced histological changes in gonadotrophic cells, liver, gonads, plasma levels of estradiol-17β and 11-ketotestosterone, and sperm motility in *Heteropneustes fossilis* (Bloch). *Chemosphere,* 72(3): 422-431.

Sitting, M., 1991. *Handbook of toxic and hazardous chemicals and carcinogens.* 3rd Ed. Noyes Publications, New Jersey.

Smith, J.F. And Dexter, D., 1963. Papillary neoplasms of the bronchus of low-grade malignancy. *Thorax,* 18: 340-349.

Steiner Pe . Carcinoma of the liver in the United States. *Acta Unio Int Contra Cancrum.* 1957;13(4-5):628-45.

Stocks, P. And Campbell, J. M., 1955. Lung cancer death rates among non-smokers and pipe and cigarette smokers; an evaluation in relation to air pollution by benzpyrene and other substances. *Br. Med. J.,* 2: 923-929.

Suralles, J., Xamena, N., Creus, A., Catalan, J., Norppa, H. And Marcos, R., 1995. Induction of micronuclei by five pyrethroid insecticides in whole blood and isolated human lymphocyte cultures. *Mutat. Res.,* 341: 169–184.

Takayasu K, Muramatsu Y, Mizuguchi Y, Moriyama N, Ojima H. Imaging of early hepatocellular carcinoma and adenomatous hyperplasia (dysplastic nodules) with dynamic ct and a combination of CT and angiography: experience with resected liver specimens. *Intervirology*. 2004;47(3-5):199-208.

Tian, H.L., 1993. Biochemical and toxicological studies of fenvalerate on the lung. *Zhonghua Yu Fang Yi Xue Za Zhi*., 27(5): 290-293.

Tomlin, C. (Ed.), 1994. *A World Compendium. The Pesticide Manual. Incorporating the agrochemical handbook.* 10th Ed. Crop Protection Publications, Bungay Suffolk, U.K.

Tuzmen, N., Candan, N., Kaya, E. And Demiryas, N., 2008. Biochemical effects of chlorpyrifos and deltamethrin on altered antioxidative defense mechanisms and lipid peroxidation in rat liver. *Cell Biochem. Funct.,* 26(1): 119-124.

United States Environmental Protection Agency, Office of Pesticides and Toxic Substances. Office of Pesticide Programs. 1989. Pesticide fact sheet. *Cypermethrin*. No. 199. Washington, D.C. (Jan. 3.)

United States Environmental Protection Agency, 1997. *Office of Pesticide Programs reference dose tracking report*. Washington, D.C.

Vineis, P. And Husgafvel-Pursiainen, K., 2005. Air pollution and cancer: biomarker studies in human populations. *Carcinogenesis,* 26(11): 1846-1855.

World Health Organization, 1989. *Environmental Health Criteria*. *Cypermethrin*. Vol. 82. United Nations Environmental Programme, the International Labour Organization, and the World Health Organization. Geneva.

World Health Organization. *WHO Recommended Classification of Pesticides by Hazard and guidelines to classification 1994–95, WHO,* Geneva , Switzerland.

Wurschmidt, F., Bunemann, H., Heilmann, H.P., 1996. Small cell lung cancer with and without superior vena cava syndrome: A multivariate analysis of prognostic factors in 408 cases. *Lung Cancer*, 14(1): 160-160.

Yavasoglu, A., Sayim, F., Uyanikgil, Y., Turgut, M. And Karabayyavasoglu, N.U., 2006. The pyrethroid cypermethrin-induced biochemical and histological alterations in rat liver. *J. Health Sci.,* 52(6): 774-780.

Zhiyong, L., Jianlin, W., Dong, Y. And Lizhi, Z., 2006. Large cell carcinoma of lung: analysis of CT signs and review of the literature. *Chin. Ger. J. Clin. Oncol.*, 5(5): 309-311.

7

The Influence of Synthetic Pyrethroids on Memory Processes, Movement Activity and Co-Ordination in Mice

Barbara Nieradko-Iwanicka
Medical University of Lublin, Chair and Department of Hygiene
Poland

1. Introduction

In plants of C*hrysanthemum* species *(Tancetum cinerariefolium, or Chrysanthemum cinerariefolium, or Pyrethrum cinerariefolium)* there are natural compounds called pyrethrins of insecticidal properties. Since the beginning of the XIXth century the flowers of C*hrysanthemum* were used to fight nets and lice. In 1828 massive production of C*hrysanthemum* began in order to make an insecticidal formulas. Before World War II the main centers of C*hrysanthemum* production were Dalmatia and Japan. After World War II production of C*hrysanthemum* was launched in Africa (Kenia, Tanzania, Rwanda). Until 1920s kerosene extract of C*hrysanthemum* flowers was commonly used to fight nets, lice and mosquitoes. Initially, it was believed to be absolutely safe for users and it was recommended to take 10-20 mg of the formula orally in adults or 5-10 mg in children to get rid of intestinal parasites. Adverse events of such use, especially skin reactions to pyrethrins were described afterwards (Casida J.E. 1980).

The chemical structure of pyrethrins was discovered by Herman Staudinger and Leopold Ruzicka between 1910 and 1916 (Casida J.E. 1980). Thre six natural pyrethrins (pyrethrin I, pyrethrin II, cinerin I, cinerin II, jasmolin I and jasmolin II) are esters of two cyclopropanecarboxylic acids and three cyclopentenolone alcohols (piretrol, jasmolol or cynerol) (Różański, 1998).

The main drawback pf pyrethrins was their instability in light and air and lack of residual activity after single use, which made them unsuitable for use in agriculture for crop protection from pest insects (Soderlund 2002).

In 1949 allethrin –the first syntheic pyrethroid was produced (Bradberry et al., 2005). It was followed by resmethrin, tetramethrin, bifenthrin and tefluthrin. Permethrin was the first pyrethroid of enough photostability to be used in agriculture. In current use there are mainly permethrin, deltamethrin, cypermethrin cyfluthrin, cyhalothrin and fenpropathrin (Soderlund 2002). These compounds exhibit strong insecticidal activity and photostability.

Since the 1970s pyrethroids have been used in public health to prevent vector born diseases like malaria (Soderlund 2002). Malaria affects people in over 110 tropical and subtropical countries. About one million people die every year because of it. Clinical symptoms of malaria develop in almost 500 million people per year (Kajfasz 2011). It is estimated, that about 15 thousand Europeans import malaria from tropical countries

every year (Knap & Myjak 2009). Current climate change is expected to have a substantial effect on the burden of mosquitoes such as anopheles species, which transmit malaria and other mosquito-borne diseases like West Nile virus infection (Shuman 2010). With global temperatures increase by 2 to 3^{o}C, the population at risk for malaria is expected to increase by 3-5%, i. e. millions of additional people would probably become infected with malaria each year (Shuman 2010). Therefore people use bednets soaked with pyrethroids or spray them indoors or outdoors to protect themselves from insect vectors carrying diseases. This means prolonged (subchronic or chronic) exposure of humans (considered to be the non-target organisms) to pyrethroid insecticides. Side effects of such uses are not fully understood and need further studies.

Pyrethroids are also commonly used in veterinary medicine, for agricultural and horticultural purposes. It was estimated, that pyrethroid use has increased to reach about 23% of the world insecticide market (Soderlund 2002). Even though organophosphates still are in wide use as insecticides (Casida & Quistad, 1998), there is evidence that they contaminate groundwaters (Badach et al., 2007; Drożdżyński 2008) , are able to accumulate in fatty tissue of living organisms (Molina et al., 2005) and can irreversibly damage the hippocampal structure in the central nervous system of mammals (Mitra et al., 2008). As hippocampus plays a key role in memory processes, it might impair memory in humans exposed to organophosphates. Therefore there is a growing interest in pyrethoids and so is their use. However, the knowledge of pyrethroids' effects on memory processes and movement activity in non-target organisms is incomplete.

Pyrethoids act as acute neurotoxins (Soderlund 2002). They alter functioning of nerves in target animals by modifying the kinetic characteristics of voltage sensitive sodium channels (Soderlund & Bloomquist, 1989; Bloomquist 1993).

Before 1970s little was known about mammalian toxicity of pyrethroids.The first study documenting modest oral toxicity of pyrethrins and pyrethoids in rats was published in 1972 (Verschoyle & Barnes, 1972). Acute mammalian toxicity of pyrethroids and structure-toxicity relationships were elucidated after numerous experiments with intracerebral and intravenous administration of these neurotoxins to mice and rats (Verschoylea & Aldridge, 1980; Lawrence & Casida,1982).

The milestone in pyrethroid improvement was the discovery of the fact that the presence of an α-cyano substituent in S configuration in the 3- phenoxybenzyl alcohol moiety greatly enhanced neurotoxicity in mammals as well as in insects (Elliot et al. 1974; Soderlund 2002). There were found pyrethroid structure-toxicity relationships in mammals, which were congruent with those found in insects (Soderlund 2002). The studies from 1970s provided the first descriptions of signs of pyrethroid intoxication in mammals after oral and intravenous dosing (Verschoyle & Barnes 1972). The authors described hypersensitivity and aggression followed by tremor, coma, an eventually death after exposure of the experiment rats to bioallethrin and resmethrin orally or intravenously (Verschoyle & Barnes 1972). The first pyrethroid containing the α-cyano-3-phenoxybenzyl moiety was deltamethrin (Elliot et al. 1974). Oral or intravenous exposure of rats to deltamethrin produced salivation, jerking leg movements and choreoatetosis (Elliot et al. 1974). In 1980 pyrethroids were divided into two groups: those producing T–syndrome (tremor) and those producing CS-syndrome (choreoatetosis and salivation) (Verschoyle & Aldridge 1980). It was found then, that majority of pyrethroids containing the α-cyano-3-phenoxybenzyl moiety produced the CS-syndrome, and those without the α-cyano-3-phenoxybenzyl moiety produced the T-syndrome.

In 1982 a new nomenclature for pyrethroids (Type I/II) was proposed basing on the syndromes of intoxication in mammals (Lawrence & Casida 1982). This nomenclature is used parallel to the T/CS nomenclature in many publications. Type I pyrethroids are considered to produce the T-syndrome, and Type II the CS-syndrome. However, there were some pyrethroids that were tested neither in the study about Type I/II nor T/CS pyrethroids, for example bifentrin and cyhalothrin. Also, in the classifying studies pyrethroids were administered orally, intravenously or intracerebrally (Verschoyle & Aldridge 1980; Lawrence & Casida 1982). However the most likely routes of pyrethroid intake by humans is orally, by inhalation and transdermally. The transdermal absorption of pyrethroids is most likely in greenhouse workers and farmers. It is also possible in holidaymakers or campers who use ' mosquito repellent' containing pyrethroids all evening or day and night. Pyrethroid containing formulas available on the market usually have enclosed leaflet warning the users about skin reactions to the chemicals and suggest symptomatic treatment if they develop. There is little or no information about their main mechanism of toxic action- about neurotoxicity.
The aim of this chapter is to analyze the influence of synthetic pyrethroids injected intraperitioneally to female Albino Swiss mice at the doe of $0.1LD_{50}$ on memory processes, movement activity and co-ordination. The pyrethroids chosen were lambda-cyhalothrin, deltamethrin, cypermethrin, fenpropathrin and bifenthrin.

2. Material and methods

2.1 Animals

Non-gravid female albino Swiss mice weighing 18-24g approximately 6 weeks of age purchased from a licensed dealer (T. Górzkowski, Warsaw, Poland) were used in the study. All animals were given a 7-day acclimation period and maintained on a 12 hr light/dark cycle. Food and tap water were provided *ad libitum.* Temperature was maintained at 21 ± 2^{o}C.

2.1.1 Groups of animals

There were two groups of animals in each experiment: I injected with 0.9% NaCl i.p. and II injected with a pyrethroid (lambda-cyhalothrin, deltamethrin, cypermethrin, fenpropathrin or bifenthrin) at the dose of $0.1LD_{50}$ i.p. The injections were made 15 min. before beginning each experiment. In passive avoidance task the injections were given once only -15 min. before training.

2.1.2 Opinion of the local ethics committee

The Local Ethics Committee for Animal Experiments in Lublin approved the experiment (Opinion No. 30/2000, dated 24.11.2000).

2.2 Materials

Bifenthrin (powder 99,6±0,2%) in glasses 0,1g each was purchased from the manufacturer – Institute of Industrial Organic Chemistry, Annopol 6, 03-236 Warsaw, Poland.
Cypermethhrin (powder 99,7%) in glasses 0,25g each was purchased from the manufacturer – Institute of Industrial Organic Chemistry, Annopol 6, 03-236 Warsaw, Poland.
Deltamethrin (powder 99,7%) in glasses 0,25g each was purchased from the manufacturer – Institute of Industrial Organic Chemistry, Annopol 6, 03-236 Warsaw, Poland.

Fenpropathrin (powder 99,4 ±0,3%) in glasses 0,25g each was purchased from the manufacturer – Institute of Industrial Organic Chemistry, Annopol 6, 03-236 Warsaw, Poland.
Lambda-cyhalothrin (Karate 025EC containing 25g of lambda-cyhalothrin per 1 litre) was purchased from the manufacturer Syngentia Limited, United Kingdom.
In order to suspend the pyrethroids in bidistilled water Tween 60 (poloxyethylene sorbitan monostearate 100% in glasses 250g each was purchased from the manufacturer – Laboratorium Reagenzien, Germany) was used. Analytic weighing scale manufactured at Radwag, Poland was used.
Water was bidistilled at the Hygiene Department of Medical University of Lublin.
0.9% NaCl for control animals was prepared at the Hygiene Department of Medical University of Lublin.

2.3 Dosing

LD_{50} of each pyrethroid was calculated with Lichtfield and Wilcoxon's method (Lichtfield & Wilcoxon 1949).
LD_{50} for bifenthrin in mice was calculated to be 16.1 mg /kg of bw [13.1-19.7].
LD_{50} for cypermethrin in mice was calculated to be 169.9 mg /kg of bw [151.9-190.1].
LD_{50} for deltamethrin in mice was calculated to be 83 mg /kg of bw [79.2-87].
LD50 for fenpropathrin in mice was calculated to be 23.8 mg /kg of bw [21.2-26.7].
LD50 for lambda-cyhalothrin in mice was calculated to be 6.9 mg /kg of bw [5.5-8.5].
At the beginning of each experiment 0.1 of LD_{50} of the tested pyrethroid was injected i.p. to each animal from group II. Controls were mice from group I, that received respective volume of 0.9% Na Cl i.p.

2.4 Behavioral tests

2.4.1 Passive avoidance

A step-through passive avoidance (PA) task was used in the study. The task relies on the innate preference of rodents for dark, enclosed spaces and it is regarded as a measure of long-term memory retention. Avoidance training consisted of a single trial in which each animal was placed in an illuminated box (15 x 12 x 15cm) adjacent to a darkened one (the same size) with an electric grid floor. A 4 x 5 cm doorway was located at floor level in the center of the wall separating the boxes. Thirty second after placing the animal in the centre of the illuminated box a passage joining the two boxes was opened. After entering the dark box the animal was punished with an electric foot shock (2 mA for 2s). Twenty four hours after the training trial memory retention test was conducted in which the same animals were placed in the illuminated box and the latency to enter the darkened box was recorded. The test ended when the mouse entered the darkened box or when 180s has elapsed. Mice that did not enter in the time allotted received latency 180s. Administration of the tested pesticide before training may impair or improve learning by affecting memory acquisition and/or recalling.

2.4.2 Y-maze task

Spontaneous alternation was assessed in a Y-maze, which is used as a measure of working spatial memory. The total number of arm entries in Y-maze can be also considered a measure of exploratory locomotor activity. The Y- maze consists of three 10 x 10 x 10 cm compartments without floor joined together with 4-cm long corridors at 120^{o} in such a way

that each corridor opens to one compartment only. The maze is placed on a clean sheet of paper on a table-top. In order to prevent odor cues, the maze was cleaned between the trials of different mice and a clean sheet of paper was used for each animal. Mice naturally tend to explore the maze by systematically entering each arm. The ability to alternate requires that the animals knew which arm they have already visited. In the task, each mice was placed at the end of one arm and was allowed to move through the maze for 8 min. The percentage of alternation, defined as consecutive entries into all three arms without repetitions in overlapping triplet sets, to all possible alternations x 100 % was counted. For example, if the arms were marked as X, Y and Z and the animal entered the arms in a following order XYZXZYZXYXYZXZ, the actual alternation would be 7, the total number of arm entries would be 14 and the percent alternation would be 58.33%.

2.4.3 Movement activity

Horizontal spontaneous locomotor activity was assessed with an automated device consisting of a circular box (32 cm in diameter) with two photocells mounted horizontally 2 cm above the floor at the angle of 90°. The photo-beam was activated when the mouse interrupted the beam. In the task the animals were not habituated for the apparatus, therefore they were placed individually in the actometers for 1 hour (two subsequent 30-min periods: 0.-30. min, 31.-60. min). The number of impulses was recorded after 30 and 60 min. The first period was considered as a rate of exploratory locomotor activity. The second period was considered as a rate of spontaneous locomotor activity.

2.4.4 Movement co-ordination

Movement co-ordination was examined on a rod rotating at the rate of 10 cycles per min. The animals were placed on the rod (1 cm diameter) 50 cm above the ground for 120 sec. The trial ended when the mouse fell off the rod or 120 s had elapsed, whichever occurred first.

2.5 Statistical analyses

A Kruskall-Wallis non- parametric ANOVA test was used to analyze the data from passive avoidance task. PA results were expressed as median values with the 25^{th} and 75^{th} percentiles. The results of spontaneous alternation in Y-maze task, movement co-ordination and spontaneous motor activity were shown as means ± SEM, and evaluated by one-way analysis of variance ANOVA followed by Student- Newman-Keuls test. The p value < 0.05 was considered statistically significant.

3. Results

3.1.1 Effect of bifenthrin on memory retention in passive avoidance task

No statistically significant difference was observed in memory retention between group I and II. Median values of latency (with the 25^{th} and 75^{th} percentiles) were: 180 sec. (180, 180) in group I (control) and 180 sec. (180, 180) in group II (0.1 LD_{50} of bifenthrin i.p.) . Post test were not calculated; $p > 0.05$. There were 10 mice in each group.

3.1.2 Influence of bifenthrin on working spatial memory in Y - maze task

There was a statistically significant difference between the examined groups in working spatial memory. Results obtained were (% of logical alternation behaviour expressed as

mean ± SEM) : group I (control) 62.2 ± 1.601; group II (0.1 LD_{50} of bifenthrin i.p.) 52.59 ± 2.932; p= 0.0116 considered significant. There were 10 mice in each group (Fig.1.).

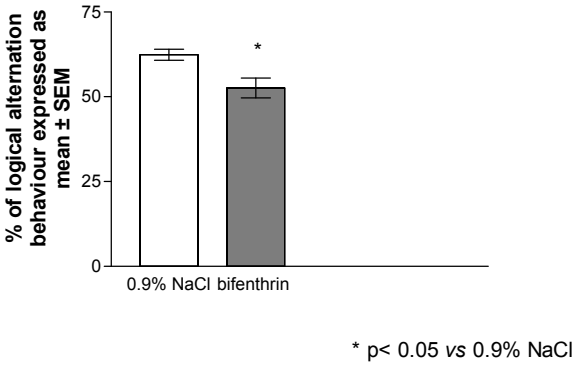


Fig. 1.The influence of bifenthrin (0.1 LD_{50} i.p.) on working spatial memory in Y- maze task. Columns represent the means ± SEM. Number of mice in each group was 10. * p < 0.05 *vs* 0.9% NaCl (ANOVA).

3.1.3 Influence of bifenthrin on movement activity in the actometer

The results of movement activity assessed within the 0-30 min. of observation were significantly different in the groups. Mean values ± SEM were: group I (control) 521.4 ± 33.903; group II (0.1 LD_{50} of bifenthrin i.p.) 664.9 ± 37.721; p= 0.0116 considered significant. There were 10 mice in each group.

The movement activity assessed within the 31-60 min. of observation did not significantly differ. Mean values ± SEM were: group I (control) 342.9 ± 39.672; group II (0.1 LD_{50} of bifenthrin i.p.) 346.6 ± 33.532.p> 0.05 considered not significant. There were 10 mice in each group (Fig.2.).

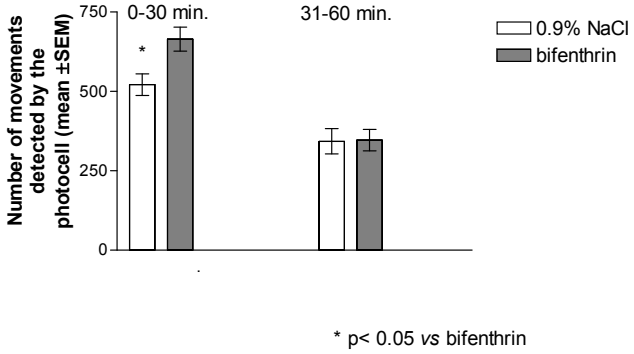
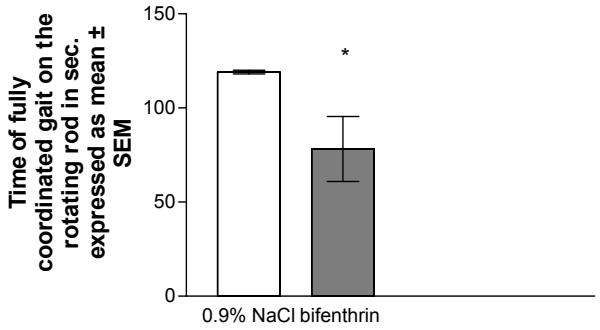


Fig. 2. The influence of bifenthrin (0.1 LD_{50} i.p.) on movement activity in the actometer. Columns represent the means ± SEM. Number of mice in each group was 10. * p< 0.05 *vs* bifenthrin (ANOVA).

3.1.4 Influence of bifenthrin on movement coordination

There was observed a statistically significant difference between the examined groups in movement coordination. The mean times of fully coordinated gait on the rotating rod in sec. (± SEM) were: group I (control) 119 ± 1.0; group II (0.1 LD_{50} of bifenthrin i.p.) 78.2 ± 17.285. p= 0.03 considered significant. There were 10 mice in each group (Fig.3).



* p< 0.05 *vs* 0.9% NaCl

Fig. 3. The influence of bifenthrin (0.1 LD_{50} i.p.) on movement coordination. Columns represent the means ± SEM. Number of mice in each group was 10. * p< 0.05 *vs* 0.9% NaCl (ANOVA).

3.2.1 Effect of cypermethrin on memory retention in passive avoidance task

No statistically significant difference was observed in memory retention between group I and II. Median values of latency (with the 25th and 75th percentiles) were: 180 sec. (180, 180) in group I (control) and 172.5 sec. (40, 180) in group II (0.1 LD_{50} of cypermethrin i.p.). Post test were not calculated; p> 0.05. There were 10 mice in each group.

3.2.2 Influence of cypermethrin on working spatial memory in Y- maze task

There was no statistically significant difference between the examined groups in working spatial memory. Results obtained were (% of logical alternation behaviour expressed as mean ± SEM): group I (control) 64.47 ± 2.361; group II (0.1 LD_{50} of cypermethrin i.p.) 59.41 ± 2.853 ; p > 0.5 considered not significant. There were 10 mice in each group.

3.2.3 Influence of cypermethrin on movement activity in the actometer

The movement activity assessed within the 0-30 min. of observation were not significantly different in the groups. Mean values ± SEM were: group I (control) 552.44 ± 66.165 ; group II (0.1 LD_{50} of cypermethrin i.p.) 368.125 ± 87.653; p >0.05 considered not significant. There were 10 mice in each group.

The movement activity assessed within the 31-60 min. of observation did not significantly differ. Mean values ± SEM were: group I (control) 394.66 ± 40.48; group II (0.1 LD_{50} of cypermethrin i.p.) 260 ± 107.11; p > 0.05 considered not significant. There were 10 mice in each group.

3.2.4 Influence of cypermethrin on movement coordination

There was observed no statistically significant difference between the examined groups in movement coordination. The mean times of fully coordinated gait on the rotating rod in sec. (± SEM) were: group I (control) 120 ± 0.0 ; group II (0.1 LD_{50} of cypermethrin i.p.) 120 ± 0.0. There were 10 mice in each group.

3.3.1 Effect of deltamethrin on memory retention in passive avoidance task

No statistically significant difference was observed in memory retention between group I and II. Median values of latency (with the 25th and 75th percentiles) were: 180 sec. (180, 180) in group I (control) and 180 sec. (65, 180) in group II (0.1 LD_{50} of deltamethrin i.p.). Post test were not calculated; $p > 0.05$. There were 10 mice in each group.

3.3.2 Influence of deltamethrin on working spatial memory in Y- maze task

There was no statistically significant difference between the examined groups in working spatial memory. Results obtained were (% of logical alternation behaviour expressed as mean ± SEM): group I (control) 61.84 ± 1.492; group II (0.1 LD_{50} of deltamethrin i.p.) 56.22 ± 3.274 ; $p > 0.5$ considered not significant. There were 10 mice in each group.

3.3.3 Influence of deltamethrin on movement activity in the actometer

The movement activity results analyzed within the 0-30 min. of observation were not significantly different in the groups. Mean values ± SEM were: group I (control) 728.66 ± 288.62; group II (0.1 LD_{50} of deltamethrin i.p.) 417 ± 50.964; $p > 0.05$ considered not significant.

The movement activity assessed within the 31-60 min. of observation did not significantly differ between the examined groups . Mean values ± SEM were: group I (control) 608.66 ± 371.16; group II (0.1 LD_{50} of deltamethrin i.p.) 215.66 ± 41.571; $p > 0.05$ considered not significant.

3.3.4 Influence of deltamethrin on movement coordination

There was observed no statistically significant difference between the examined groups in movement coordination. The mean times of fully coordinated gait on the rotating rod in sec. (± SEM) were: group I (control) 112.2 ± 7.8 ; group II (0.1 LD_{50} of deltamethrin i.p.) 82 ± 15.721 ; $p > 0.05$ considered not significant. There were 10 mice in each group.

3.4.1 Effect of fenpropathrin on memory retention in passive avoidance task

No statistically significant difference was observed in memory retention between group I and II. Median values of latency (with the 25th and 75th percentiles) were: 180 sec. (180, 180) in group I (control) and 30 sec. 135, 180) in group II (0.1 LD_{50} of fenpropathrin i.p.). Post test were not calculated. There were 10 mice in each group.

3.4.2 Influence of fenpropathrin on working spatial memory in Y- maze task

There was no statistically significant difference between the examined groups in working spatial memory. Results obtained were (% of logical alternation behaviour expressed as mean ± SEM): group I (control) 62.38 ± 1.709; group II (0.1 LD_{50} of fenpropathrin i.p.) 61.88 ± 3.379 ; $p > 0.5$ considered not significant. There were 10 mice in each group.

3.4.3 Influence of fenpropathrin on movement activity in the actometer

The results of movement activity measurement analyzed within the 0-30 min. of observation were not significantly different in the examined groups. Mean values ± SEM were: group I (control) 524.44 ± 64.61 ; group II (0.1 LD_{50} of fenpropathrin i.p.) 456 ± 45.128; $p > 0.05$ considered not significant.

The movement activity assessed within the 31-60 min. of observation did not significantly differ between the examined groups . Mean values ± SEM were: group I (control) 360 ± 51.465 ; group II (0.1 LD_{50} of fenpropathrin i.p.) 316.77 ± 34.44 ; $p > 0.05$ considered not significant. There were 10 mice in each group.

3.4.4 Influence of fenpropathrin on movement coordination

There was observed no statistically significant difference between the examined groups in movement coordination. The mean times of fully coordinated gait on the rotating rod in sec. (± SEM) were: group I (control) 112.3 ± 7.7 ; group II (0.1 LD_{50} of fenpropathrin i.p.) 105.8 ± 9.881 ; $p > 0.05$ considered not significant. There were 10 mice in each group.

3.5.1 Effect of lambda-cyhalothrin on memory retention in passive avoidance task

No statistically significant difference was observed in memory retention between group I and II. Median values of latency (with the 25^{th} and 75^{th} percentiles) were: 180 sec. (180, 180) in group I (control) and 180 sec. (15, 180) in group II (0.1 LD_{50} of lambda-cyhalothrin i.p.). $p > 0.05$ considered not significant. Post test were not calculated. There were 10 mice in each group.

3.5.2 Influence of lambda-cyhalothrin on working spatial memory in Y- maze task

There was no statistically significant difference between the examined groups in working spatial memory. Results obtained were (% of logical alternation behaviour expressed as mean ± SEM): group I (control) 62.57 ± 2.875; group II (0.1 LD_{50} of lambda-cyhalothrin i.p.) 61.83 ± 1.865; $p > 0.5$ considered not significant. There were 10 mice in each group.

3.5.3 Influence of lambda-cyhalothrin on movement activity in the actometer

The movement activity results analyzed within the 0-30 min. of observation were not significantly different in the examined groups. Mean values ± SEM were: group I (control) 546.8 ± 28.171 ; group II (0.1 LD_{50} of lambda-cyhalothrin i.p.) 491.9 ± 28.917; $p > 0.05$ considered not significant.

The movement activity assessed within the 31-60 min. of observation did not significantly differ between the examined groups. Mean values ± SEM were: group I (control) 357.8 ± 31.48 ; group II (0.1 LD_{50} of lambda-cyhalothrin i.p.) 291.4 ± 22.935 ; $p > 0.05$ considered not significant. There were 10 mice in each group.

3.5.4 Influence of lambda-cyhalothrin on movement coordination

There was observed no statistically significant difference between the examined groups in movement coordination. The mean times of fully coordinated gait on the rotating rod in sec. (± SEM) were: group I (control) 119 ± 1.0; group II (0.1 LD_{50} of lambda-cyhalothrin i.p.) 107.6 ± 9.48 ; $p > 0.05$ considered not significant. There were 10 mice in each group.

4. Discussion

In this work bifenthrin administered i.p. at the dose of 0.1 LD_{50} was the only pesticide tested, that produced significant changes in mice bahaviour. Bifenthrin administered once 15 min. before testing in the Y-maze has significantly impaired the spatial working memory in comparison with the control group (Fig.1.). Bifenthrin was also found to impair movement coordination (Fig.3.). Bifenthrin has significantly increased movement activity within 0-30 min. of examination in the actometer (Fig.2.). In the previous acute neurotoxicity studies bifenthrin administered orally to male and female rats at the dose of 75mg/kg bw was found to produce whole body tremors, twitching, staggered gait, uncoordinated movement, ataxia, splayed hindlimbs, abnormal posture, clonic convulsions and abdominogenital staining (Watt 1998). Bifenthrin is an non-cyano pyrethroid, which was not included in the original studies estabilishing the T/CS classification. At present bifenthrin is classified as Type I (producing T-syndrome) (Breckendridge et al. 2009). All the above data show its toxicity and suggest possibe side effects of use in non-target organisms, like humans.

Cypermethrin administered i.p. at low dose in this work to female mice did dot significantly affect memory and movement. In previous studies cypermethrin was identified as producing the CS syndrome (Verschoyle & Aldridge 1980; Lawrence & Casida 1982). In experiments with male and female rats given 100 or 200 mg of cypermethrin /kg bw in corn oil salivation, oral discharge, abdominogenital staining, ataxia, staggered gait, decreased locomotor activity and mortality were observed (Freeman 1993).

Deltamethrin did not affect movement non memory in this set of experiments. Deltamethrin is classified as producing the CS intoxication syndrome (Barnes & Verschoyle 1974).The acute neurotoxicity studies of deltamethrin were conducted on male and female rats (Nemec 1998). Deltamethrin was administered orally in corn oil at the dose of 50mg/kg bw. Deltamethrin administered this way produced salivation, a flattened posture, limb extension, clonic and tonic convulsions, tremor, biting the cage, eyelid ptosis, decreased reaction to removal and handling, lacrimation, decreased arousal, wave-like movements of the abdomen dragging hindlimbs, decreased response to stimuli, increased auditory startle response, reduced forelimb strength, decreased fore- and hindlimb extensor strength, hypothermia and mortality (Nemec 1998).

Lambda-cyhalothrin did not produce any statistically significant effect on memory processes nor movement activity in mice tested in this experiment. Cyhalothrin is a member of the α-cyano-3-phenoxybenzyl subfamily of pyrethroids (Soderlund 2002). However, it was not included in the T/CS classification as the effect of acute intoxication with lambda-cyhalothrin were not specific to any of theses types. Lambda-cyhalothrin administered orally at the dose of 35mg/kg bw in corn oil to rats caused decreased activity, ataxia, reduced stability, tiptoe gait, decreased landing foot splay and decreased tail flick response (Barmmer 1999).

There is data, that pyrethroids (cypermethrin, deltamethrin) administered orally in corn oil to rats produce dose-dependent decrease in motor activity (Crofton & Reiter 1984, 1988 a,b).

There was evidence that i.p. administration of deltamethrin to rats caused a dose-dependent reduction in frequency of a prevuoisly learned behaviour (Bloom et al. 1983; Stein et al. 1987) which is congruent with data obtained in this work. Deltamethrin administered orally to rats for 15 days reduced learning and memory measured in a Y- maze task (Husain et al. 1996). Oral administration of cypermethrin to rats caused a similar reduction in learned bahaviour (Peele & Crofton 1987).

Fenpropathrin does not fit into the traditional classification of pyrethroids (T/CS). In our former study (Nieradko-Iwanicka & Borzęcki 2010) fenpropathrin together with transient incomplete brain ischemia were found to reduce latency in the passive avoidance task compared to controls.

5. Conclusion

Pyrethroids, especially bifenthrin, should be used with caution as insecticidal formulas containing it may impair memory and movement in non target animals.

6. References

Badach H. et al. (2007) Pesticide content in drinking water samples collected from orchard areas in central Poland. *Ann Agric Environ Med*, 14, pp. 109-114.

Barmmer A. (1999) Lambda-cyhalothrin: acute neurotoxicity study in rats. Zeneca Agricultural Products Laboratory Study No. CTL/P/6151: AR 6699.

Barnes J.M., Verschoyle R.D. (1974). Toxicity of new pyrethroid insecticide. *Nature* 248, pp. 711.

Bloom A.S., Staatz C.G., Dieringer T. (1983) Pyrethroid Effects on operant responding and condiotioning) *Neurobehav Toxicol Teratol* 5, pp. 321-324.

Bloomquist J.R. (1993) Neuroreceptor mechanisms in pyrethroid mode of action and resistance In: *Reviews in Pesticide Toxicology* Roe, M., KuhrR.J. (Eds.), , Toxicology Communications, Raleigh, NC, pp. 181-226.

Bradberry S.M., et al. (2005) Poisonong due to pyrethroids. *Toxicol Rev*, 24 (2)pp. 93-106.

Breckendridge C.B., Holden L., Struggerss N., Weiner M., Sheets L., Sargent D., Soderlund D.M., Choi J.-S., Symington S., Clark J.M., Burr S., Ray D. (2009) Evidence for separate mechanism of toxicity for the Type I and the Type II pyrethroid insecticides. *Neurotocicology*, 30, pp.17-31.

Casida J.E et al. (1980) Metabolic chemistry of pyrethroid insecticides. *Pestic Sci,* 11, pp. 257-269.

Casida J.E., Quistad G.B. (1998) Golden age of insecticide research: past, present, or future? *Annu Rev Entomol* 43, pp.1-16.

Crofton K.M., Reiter L.W. (1984) Effects of two pyrethroid insecticides on motor activity and acoustic startle response in the rat. *Toxicol Appl Phasrmacol* 75, pp. 318-328.

Crofton K.M., Reiter L.W. (1988a) The effects of Type I and II pyrethroids on motor activity and acoustic startle response in the rat. *Fund Appl Toxicol*, 10, pp. 624-634.

Crofton K.M., Reiter L.W. (1988b) Pyrthroid insecticides and gamma-aminobutyric acid receptor complex. Mptor activity and acoustic startle response in the rat. *J Pharmacol Exp Ther* 243, pp. 946-954.

Drożdzyński D. (2008) Studies on residues of pesticides used in rape plants protection in surface waters of intensively exploited arable lands in Wielkopolska Province of Poland. *Ann Agric Environ Med*, 15, pp. 231-235.

Elliot M., Farnham A.W., Janes N.F., Needham P.H., Pulman D.A., (1974) Synthetic insecticide with a new order of activity. *Nature* 248, pp.710-711.

Freeman C. (1993) Cypermethrin technical: acute neurotoxicity screen in rats. FMC Corporation study No. A92-3542.

Husain R., Adhami V.M., Seth P.K. (1996) Behavioral, neurochemical, and neuromorphological effects of deltamethrin in adult rats. *J Toxicol Environ Health* 48, pp. 515-526.

Kajfasz P. (2011) (Pol.) Profilaktyka chorób tropikalnych. *Medycyna po Dyplomie* 6 (18)3, pp. 34-41.

Knap J.P & Myjak P. (2009) (Pol.) Malaria w Polsce i na świecie- wczoraj i dziś. α-medica Press Wydanie II (uzupełnione). Bielsko-Biała , pp.36.

Lawrence L.J., Casida J.E. (1982) Pyrethroid tocxicology: Mouse intracerebral structure-toxicity relationships. *Pestic Biochem Physiol*, 18, pp.9-14.

Lichtfield J.T., Wilcoxon F.A. (1949) Simplified method of evaluating dose-effect experiments. *J Pharmacol Exp Ther*, 96. pp.99-113.

Mitra N.K. et al.(2008) Evaluation of neurotoxicity of repeated dermal application of chloprioryfos on hippocampus of adult mice. *Ann Agric Environ Med*, 15,pp. 211-216.

Molina C. et al.(2005) HCH and DDT residues in human fat in the population of Murcia (Spain). *Ann Agric Environ Med*, 12 , pp. 133-136.

Nemec M (1998) An acute neurotoxicity study of deltamethrin in rats AgroEvo USA Company Study No. 53014.

Nieradko-Iwanicka B., Borzęcki A. (2010) Infl;uence of fenpropathrin on memory and movement in mice after transient incomplete cerebral ischemia. *J Toxicol Environ Health* A 73, pp. 1166-1172.

Peele D.B., crofton K.M. (1987) Pyrethroid effects on schedule- controlled behavior: time and dosage relationships. *Neurotoxicol Teratol* 9, pp. 387-394.

Różański L. (1998) (Pol.) *Przemiany pestycydów w organizmach żywych i środowisku*. AGRA-ENVIRO LAB. Poznań.

Shuman E.K. (2010) Global climate change and infectious diseases. *N Engl J Med,* 362; 12, pp. 1061-1063.

Soderlund D.M., Bloomquist J.R. (1989) Neurotoxic actions of pyrethroid insecticides. *Annu Rev Entiomol*, 34, pp.77-96.

Soderlund D.M. et al. (2002) Mechanisms of pyrethroid neurotoxicity: implications for cumulative risk assessment. *Toxicology,* 171, pp. 3-59.

Stein E.A., Washburn M., Walczak C., Bloom A.S. (1987) Effects of pyrethroid insecticides on operant responding maintained by food. *Neurotoxicol Teratol* 9, pp. 27-31.

Verschoyle R.D., Aldridgwe W.N. (1980) Structure-activity relationships of some pyrethroids in rats. *Arch Toxicol* 45, pp. 325-329.

Verschoyle R.D, Barnes J.M. (1972) Toxicity of natural and synthetic pyrethrins to rats . *Pestic Biochem Physiol*, 2, pp. 308-311.

Watt B. (1998) FMC 548000 technical: acute neurotoxicity screen in rats. FMC Corporation Study No. A97-4642.

8

Metabolism of Pesticides by Human Cytochrome P450 Enzymes *In Vitro* – A Survey

Khaled Abass[1,2*], Miia Turpeinen[1], Arja Rautio[2],
Jukka Hakkola[1] and Olavi Pelkonen[1]
[1]*Department of Pharmacology and Toxicology,*
Institute of Biomedicine, University of Oulu,
[2]*Centre of Arctic Medicine, Thule Institute,*
University of Oulu,
Finland

1. Introduction

Cytochrome P450 enzymes (CYPs) are active in the metabolism of wide variety of xenobiotics. The investigation of the contributions of human CYPs in pesticides metabolism, especially insecticides, is still growing. One of the background tools to facilitate this task is by sorting the contribution of each human CYP isoform in the metabolism of pesticides. This paper attempts to provide a comprehensive literature survey on the role of human hepatic CYPs such as human CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5 and CYP3A7 in pesticides biotransformation in vitro as well as to sort the reactions mediated. Based on relevant publications identified by searching databases from 1995 through 2011, more than 400 metabolic reactions were reported to be mediated at least in part by human CYPs in vitro. Some information on older papers was obtained from previous literature surveys compiled by Hodgson 2001 & 2003. Finally, we give brief insight into potential modulations and consequences of human CYP genes – pesticides interactions.

2. Xenobiotic biotransformation

Xenobiotic biotransformation is the process by which lipophilic foreign compounds are metabolized through enzymatic catalysis to hydrophilic metabolites that are eliminated directly or after conjugation with endogenous cofactors via renal or biliary excretion. These metabolic enzymes are divided into two groups, Phase I and Phase II enzymes (Rendic and Di Carlo, 1997; Oesch et al. 2000). Phase I reactions are mediated primarily by cytochrome P450 family of enzymes, but other enzymes (e.g. flavin monooxygenases, peroxidases, amine oxidases, dehydrogenases, xanthine oxidases) also catalyze oxidation of certain functional groups. In addition to the oxidative reactions there are different types of

* Corresponding author email: khaled.megahed@oulu.fi; khaled.m.abass@gmail.com
Permanent address: Department of Pesticides, Menoufiya University, Menoufiya, Egypt

hydrolytic reactions catalysed by enzymes like carboxylesterases and epoxide hydrolases (Low, 1998; Hodgson and Goldstein, 2001; Parkinson, 2001).

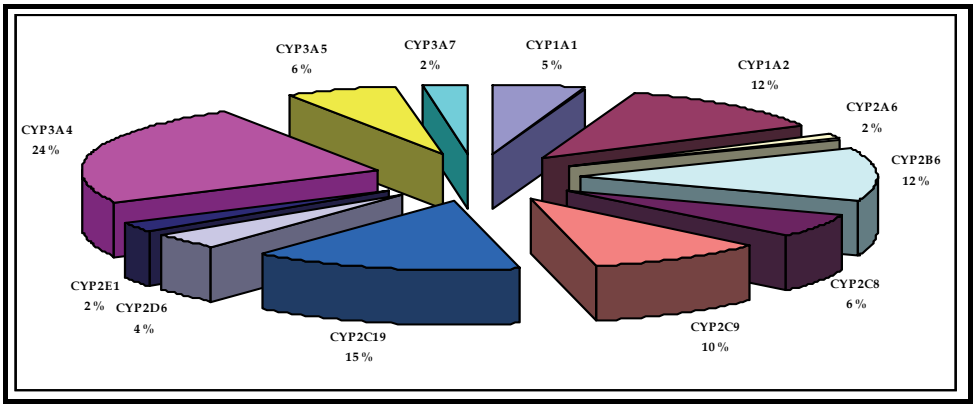


Fig. 1. The percentage of human recombinant cytochrome P450 isoforms involved in pesticides metabolism. 63 compounds (36 insecticides; 14 fungicides; 10 herbicides; 2 plant growth regulators and a biocide agent) were metabolized at least in part by one or more human enzymes yielded 495 metabolic reactions.

Phase I products are not usually eliminated rapidly, but undergo a subsequent reaction in which an endogenous substrate such as glucuronic acid, sulfuric acid, acetic acid, or an amino acid combines with the existing or newly added or exposed functional group to form a highly polar conjugate to make them more easily excreted (LeBlanc and Dauterman, 2001; Rose and Hodgson, 2004; Zamek-Gliszczynski et al. 2006).

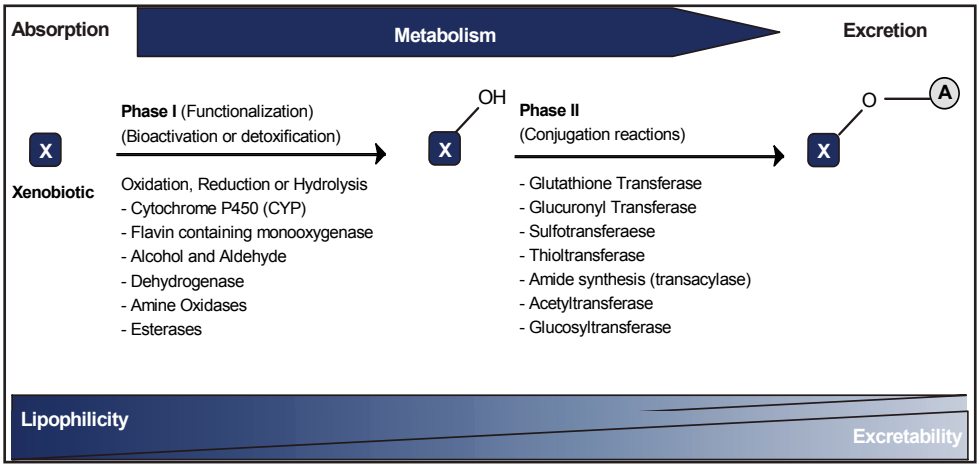


Fig. 2. Schematic description of the two main phases of drug metabolism. In general, a parent compound is converted into an intermediate metabolite which is then conjugated, but metabolism may involve only one of these reactions. Some metabolites are more toxic than the parent compound (Ahokas and Pelkonen, 2007; Liska et al. 2006).

3. Cytochrome P450 enzyme system

3.1 Nomenclature, location and microsomal preparation

P450 enzymes are categorized into families and subfamilies by their sequence similarities. The human genomes comprise 57 CYP genes and about the same numbers of pseudogenes, which are grouped according to their sequence similarity into 18 families and 44 subfamilies. The web site, http://drnelson.utmem.edu/CytochromeP450.html, contains more detailed classification related to the cytochrome P450 metabolizing enzymes. The CYP enzymes in the families 1-3 are active in the metabolism of a wide variety of xenobiotics including drugs (Rendic and Di Carlo, 1997; Pelkonen et al. 2005; Zanger et al. 2008). CYPs are found in high concentration in the liver, but are present in a variety of other tissues, including lung, kidney, the gastrointestinal tract, nasal mucosa, skin and brain (Lawton et al. 1990; Hjelle et al. 1986; Tremaine et al. 1985; Dutcher and Boyd, 1979; Peters and Kremers, 1989; Adams et al. 1991; Eriksson and Brittebo, 1991; Khan et al. 1989; Dhawan et al. 1990; Bergh and Strobel, 1992) and located primarily in the endoplasmic reticulum.

Microsomes can be prepared easily from frozen liver tissue, and enzymatic activities are stable during prolonged storage (Beaune et al. 1986; Pearce et al. 1996; Yamazaki et al. 1997). Microsomes consist of vesicles of the hepatocyte endoplasmic reticulum and are prepared by standard differential ultracentrifugation (Pelkonen et al. 1974). Microsomes are derived from the endoplasmic reticulum as a result of tissue homogenization and are isolated by two centrifugation steps. The tissues are typically homogenized in buffer and centrifuged at 10.000g for 20 minutes, the resulting supernatant, referred to as S9 fraction, can be used in studies where both microsomal and cytosolic enzymes are needed. S9 fraction is centrifuged at 100.000g for 60 minutes to yield the microsomal pellets and a cytosolic supernatant. The pellet is typically re-suspended in a volume of buffer and stored at -70° C (Figure 3) (Testa and Krämer, 2005).

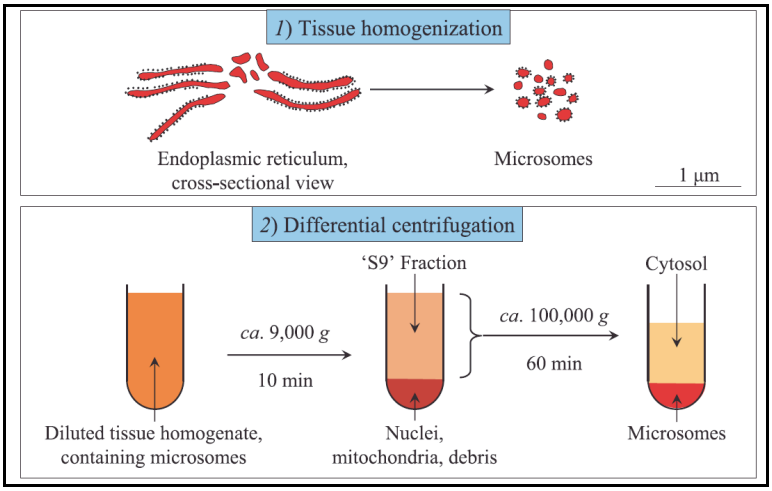


Fig. 3. A simplified scheme of the preparation of microsomes (Testa and Krämer, 2006).
Testa and Krämer: The biochemistry of drug metabolism - an introduction part 1. Principals and overview. Chemistry & Biodiversity. 2005, 3, 1053-1101; © Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

Microsomes have many advantages including easy adaptation to higher throughput assays, easy preparation and use, good stability during storage, high CYP concentration and high rate of metabolite turnover. (Pelkonen et al. 2005; Brandon et al. 2003; Ekins et al. 1999; Ekins et al. 2000; Pelkonen and Raunio, 2005).

3.2 Function

CYP oxidation reactions involve a complex series of steps. The initial step involves the binding of a substrate to oxidized CYP, followed by a one-electron reduction catalyzed by NADPH cytochrome P450 reductase to form a reduced cytochrome-substrate complex. The next several steps involve interaction with molecular oxygen, the acceptance of the second electron from NADPH cytochrome b5 reductase, followed by subsequent release of water and the oxygenated product of the reaction. This reaction sequence results in the addition of one oxygen atom to the substrate, while the other atom is reduced to water (Parkinson, 2001; Rose and Hodgson, 2004; Guengerich, 2001) (figure 3).

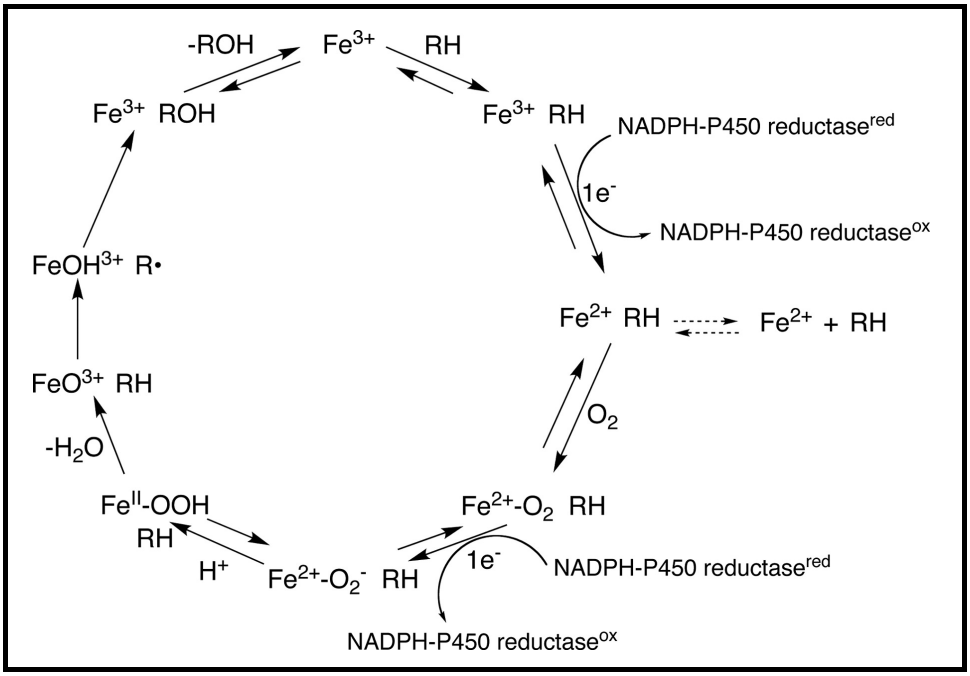


Fig. 4. Generalized P450 catalytic cycle (Sohl et al. 2008) (Sohl et al. J. Biol. Chem. 2008).

4. *In vitro* approaches

In vitro approaches to characterize metabolic fate for human clearance predication have become more frequent with the increase in the availability of human-derived materials. All

models have certain advantages and disadvantages, but the common advantage to these approaches is the reduction of the complexity of the study system. In vitro model range from simple to more complex systems: individual enzymes, subcellular fractions, cellular systems, liver slices and whole organ, respectively. However, the use of in vitro models is always a compromise between convenience and relevance. Different in vitro models and their advantages and disadvantages have been described previously (Pelkonen et al. 2005; Brandon et al. 2003; Pelkonen and Raunio, 2005; Pelkonen and Turpeinen, 2007).

5. Identification of the individual CYP enzyme(s) involved in the metabolism of a xenobiotic

To understand some of the factors related to xenobiotic metabolism that can influence the achievement of these aims, there are several important points to consider such as determination of the metabolic stability of the compound, identification of reactive metabolites, evaluation of the variation between species, identification of human CYPs and their isoforms involved in the activation or detoxification, evaluation of the variation between individuals, identification of individuals and subpopulations at increased risk and finally overall improvement of the process of human risk assessment.

Basically the identification of the individual CYP enzyme(s) involved in the metabolism of a xenobiotic is necessary for in vitro – in vivo extrapolation and prediction if the results of the metabolic stability and metabolic routes in human in vitro systems indicate that CYP enzymes contribute significantly to the metabolism of a xenobiotic. Due to the broad substrate specificity of CYP enzymes, it is possible for more than one enzyme to be involved in the metabolism of a single compound.

In vitro methods have been established to determine which CYP isoform(s) is (are) involved in the metabolism of a xenobiotic (Pelkonen et al. 2005; Pelkonen and Raunio, 2005). The identification could be achieved by different approaches such as *cDNA*-expressed enzymes, correlation studies, inhibition studies with CYP-selective chemical inhibitors and specific antibodies and inhibition of CYP enzymes.

5.1 *cDNA*-expressed enzymes

The availability of a full panel of recombinant enzymes covering the major human liver CYPs allows a direct approach for assaying the metabolism of a compound by incubation with the isolated isoforms. This can be done by following substrate consumption or product formation by each isoform using the same analytical methods as for human liver microsomes-based assays (Reponen et al. 2010). The biotransformation of a xenobiotic by a single CYP does not necessarily mean its participation in the reaction in vivo. The relative roles of individual CYPs cannot be quantitatively estimated using this approach due to the interindividual variation in the levels of individual active CYPs in the liver (Guengerich, 1999; Guengerich, 1995). However, cDNA-expressed CYPs are well suited for isozyme identification in a high-throughput screening format (White, 2000). The relative importance of individual isoform to in vivo clearance is dependent upon the relative abundance of each isoform. When taking into account the average composition of human hepatic CYPs, an approximate prediction of the participation of any CYP enzyme in the whole liver activity can be achieved (Rodrigues, 1999; Rostami-Hodjegan and Tucker, 2007).

5.2 Correlation studies

Using a bank of "phenotyped" liver microsomes, correlation analysis could be performed. Correlation analysis involves measuring the rate of xenobiotic metabolism by several liver samples from individual humans and correlating reaction rates with the level of activity of the individual CYP enzymes in the same microsomal samples. If there are a sufficient number of individual samples (at least ten), the correlation plot would give the information needed for the evaluation of the participating CYPs. The higher the correlation between the activities, the larger the probability that the respective CYP enzyme is responsible for the metabolism of the xenobiotic. Another approach is to correlate the levels of an individual CYP determined by Western blot analysis against the metabolic activity (Beaune et al. 1986; Brandon et al. 2003; Berthou et al. 1994; Guengerich, 1995; Jacolot et al. 1991; Wolkers et al. 1998).

5.3 Inhibition studies with CYP-selective chemical inhibitors and specific antibodies

Pooled human liver microsomes or individual liver microsomal samples should be used to examine the effect of CYP-selective chemical inhibitors or selective inhibitory antibodies. Antibody inhibition involves an evaluation of the effects of inhibitory antibodies against selective CYP enzymes on the metabolism of a xenobiotic in human liver microsomes. Chemical inhibition involves an evaluation of the effects of known CYP enzyme inhibitors on the metabolism of a xenobiotic. Several compounds have been characterized for their inhibitory potency against different CYPs; for example, furafylline is perhaps the most potent and selective inhibitor of CYP1A2, tranylcypromine of CYP2A6, thiotepa and ticlopidine of CYP2B6, trimethoprim and sulfaphenazole are selective inhibitors of CYP2C8 and CYP2C9, respectively, fluconazole may be used for CYP2C19, quinidine is a commonly used in vitro diagnostic inhibitor of CYP2D6 activity, pyridine and disulfiram of CYP2E1, and ketoconazole and itraconazole are among many potent and relatively selective inhibitors of CYP3A4 often used in vitro and in vivo as diagnostic inhibitors (Rendic and Di Carlo, 1997; Pelkonen et al. 2005; Pelkonen and Raunio, 2005; Bourrie et al. 1996; Clarke et al. 1994; Nebert and Russell, 2002; Pelkonen et al. 2008; Schmider et al. 1995; Sesardic et al. 1990).

5.4 Inhibition of CYP enzymes

Testing the inhibitory interactions of a xenobiotic on CYP-specific model activity in human liver microsomes in vitro provides information about the affinity of the compound for CYP enzymes (Pelkonen and Raunio, 2005). The type of CYP inhibition can be either irreversible (mechanism-based inhibition) or reversible. Irreversible inhibition requires biotransformation of the inhibitor, while reversible inhibition can take place directly, without metabolism. Reversible inhibition is the most common type of enzyme inhibition and can be further divided into competitive, noncompetitive, uncompetitive, and mixed-type inhibition (Pelkonen et al. 2008). The inhibitory interactions of a xenobiotic on CYP enzymes can be tested by co-incubating a series of dilutions of a xenobiotic with a reaction mixture containing single or multiple substrates. In the single substrate assay, traditionally CYP interaction studies are performed using specific assays for each CYP isoform. A decrease in probe metabolite formation produced by inhibition is usually analyzed by LC-UV, LC-MS or fluorometry. In the cocktail assay, several CYP-selective probes are incubated with human liver microsomes and analyzed by LC-MS-MS (Tolonen et al. 2007; Turpeinen et al. 2006; Turpeinen et al. 2005; Tolonen et al. 2005).

6. Pesticides reported to be metabolized at least in part by certain human cytochrome P450

During the recent years, a large number of papers have been published on the activities of human CYPs involved in the metabolism of pesticides. Human CYPs involved in metabolism of pesticides and related compounds were listed and updated previously several years ago by Hodgson 2001 & 2003 (Hodgson, 2001; 2003). Abbreviations used in the coming tables are listed in table 1. The updated human CYPs and their isoforms catalyzing pesticides biotransformation in addition to reactions detection methods are listed below in tables containing the primary CYP-specific information (Tables 2 to 13). Additional summary table contains information classified according to individual metabolic reactions and chemical classes of pesticides (Table 14).

Chemical class	Abb.	Pesticide type	Abb.	Detection method	Abb.
Acylalanine	AcA	Algicide	A.	Acetylcholine esterase inhibition	AChE inh.
Carbamates	CA	Biocide agent	B. A.	Electron capture detector	ECD
Chloroacetamide	ChAc	Biocide	B.	Gas chromatography	GC
Chlorinated cyclodiene	CCD	Fungicide	F.	Liquid chromatography	LC
Conazole	CZ	Herbicide	H.	Mass spectrometry	MS
Neonicotinoid	NC	Insect repellent	I. R.	Nuclear magnetic resonance	NMR
Organochlorine	OC	Insecticide	I.	Photo Diode Array Detector	PDA
Organophosphorus	OP	Molluscicide	M.	Thin layer chromatography	TLC
Organotin	OT	Plant growth regulator	PGR.	Ultraviolet detector	UV
Oxathiin	OX				
Phenyl pyrazole	PP				
pyrethroid	PY				
phenyl urea	PU				
Triazine	TA				
Triazole	TriA				

Table 1. Abbreviations

6.1 CYP1A1

Pesticide	Chemical class	Type	Metabolic pathway	Detection method	Reference
Ametryne	TA	H.	N-Deethylation N-Deisopropylation Sulfoxidation	LC-UV	Lang et al. 1997
Atrazine	TA	H.	N-Deethylation N-Deisopropylation	LC-UV	Lang et al. 1997
				LC/PDA & LC-MS	Joo et al. 2010
Carbaryl	CA	I.	Aromatic hydroxy-lation Methyl Oxidation	LC-UV	Tang et al. 2002
Carbosulfan	CA	I.	N-S cleavage Sulfoxidation	LC-MS	Abass et al. 2010
cis-Permethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009

DEET		I. R.	Aromatic methyl oxidation	LC-UV	Usmani et al. 2002
Dimethoate	OP	I.	Desulfuration	AChE inhibition	Buratti and Testai, 2007
Diuron	PU	H.	N-Demethylation	LC-MS	Abass et al. 2007c
Fenthion	OP	I.	Sulfoxidation	LC-UV	Leoni et al. 2008
Furametpyr	OX	F.	N-Demethylation	TLC, NMR, MS	Nagahori et al. 2000
Sulprofos	OP	I.	Sulfoxidation	LC-UV	Usmani et al. 2004b
Terbuthylazine	TA	H.	N-Deethylation	LC-UV	Lang et al. 1997
Terbutryne	TA	H.	N-Deethylation	LC-UV	Lang et al. 1997
Terbutryne	TA	H.	Sulfoxidation	LC-UV	Lang et al. 1997
τ-Permethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
β-Cyfluthrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
λ-Cyhalothrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009

Table 2. Pesticides reported to be metabolized at least in part by human CYP1A1.

6.2 CYP1A2

Pesticide	Chemical class	Type	Metabolic pathway	Detection method	Reference
Ametryne	TA	H.	N-Deethylation N-Deisopropylation Sulfoxidation	LC-UV	Lang et al. 1997
Atrazine	TA	H.	N-Deethylation N-Deisopropylation	LC-UV	Lang et al. 1997
				LC/PDA & LC-MS	Joo et al. 2010
Azinophos methyl	OP	I.	Desulfuration	AChE Inh. LC-UV	Buratti et al. 2002
Bioresmethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
Carbaryl	CA	I.	Aromatic hydroxy-lation Methyl Oxidation	LC-UV	Tang et al. 2002
Carbofuran	CA	I.	Ring oxidation	LC-UV	Usmani et al. 2004a
Carbosulfan	CA	I.	N-S cleavage	LC-MS	Abass et al. 2010
Chlorpyrifos	OP	I.	Desulfuration	AChE Inh., LC-UV	Buratti et al. 2002
			Desulfuration Dearylation	LC-UV	Tang et al. 2001; Foxenberg et al. 2007; Mutch and Williams, 2006
cis-Permethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
Cypermethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
Diazinon	OP	I.	Desulfuration	AChE Inh. LC-UV	Buratti et al. 2002
			Desulfuration Dearylation	LC-UV	Mutch and Williams, 2006; Kappers et al. 2001
Dimethoate	OP	I.	Desulfuration	AChE Inh.	Buratti and Testai, 2007
Disulfoton	OP	I.	Sulfoxidation	LC-UV	Usmani et al. 2004b
Diuron	PU	H.	N-Demethylation	LC-MS	Abass et al. 2007c

Fenthion	OP	I.	Desulfuration Sulfoxidation	LC-UV	Leoni et al. 2008
Furametpyr	OX	F.	N-Demethylation	TLC, NMR & MS	Nagahori et al. 2000
Imidacloprid	NC	I.	Nitroimine reduction	TLC	Schulz-Jander and Casida, 2002
Malathion	OP	I.	Desulfuration	AChE Inh.	Buratti et al. 2005
Methiocarb	OP	I.	Sulfoxidation	LC-UV	Usmani et al. 2004b
Methoxychlor	OC	I.	O-Demethylation	TLC	Stresser and Kupfer, 1998
Parathion	OP	I.	Desulfuration	AChE Inh., LC-UV	Buratti et al. 2002
Parathion	OP	I.	Desulfuration	AChE Inh.	Sams et al. 2000
			Desulfuration Dearylation	LC-UV	Foxenberg et al. 2007; Mutch and Williams, 2006; Mutch et al. 2003; Mutch et al. 1999; Butler and Murray, 1997
Phorate	OP	I.	Sulfoxidation	LC-UV	Usmani et al. 2004b
Sulprofos	OP	I.	Sulfoxidation	LC-UV	Usmani et al. 2004b
Terbuthylazine	TA	H.	N-Deethylation	LC-UV	Lang et al. 1997
Terbutryne	TA	H.	N-Deethylation Sulfoxidation	LC-UV	Lang et al. 1997
τ-Permethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
β-Cyfluthrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
λ-Cyhalothrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009

Table 3. Pesticides reported to be metabolized at least in part by human CYP1A2.

6.3 CYP2A6

Pesticide	Chemical class	Type	Metabolic pathway	Detection method	Reference
Carbaryl	CA	I.	Aromatic hydroxyl-lation Methyl Oxidation	LC-UV	Tang et al. 2002
Carbosulfan	CA	I.	N-S cleavage	LC-MS	Abass et al. 2010
DEET		I. R.	N-Deethylation	LC-UV	Usmani et al. 2002
Diazinon	OP	I.	Desulfuration Dearylation	LC-UV	Kappers et al. 2001
Dimethoate	OP	I.	Desulfuration	AChE Inh.	Buratti and Testai, 2007
Diuron	PU	H.	N-Demethylation	LC-MS	Abass et al. 2007c
Imidacloprid	NC	I.	Imidazolidine oxidation	TLC	Schulz-Jander and Casida, 2002

Table 4. Pesticides reported to be metabolized at least in part by human CYP2A6.

6.4 CYP2B6

Pesticide	Chemical class	Type	Metabolic pathway	Detection method	Reference
Acetachlor	ChAc	H.	N-Dealkoxylation	LC-UV	Coleman et al. 2000
Alachlor	ChAc	H.	N-Dealkoxylation	LC-UV	Coleman et al. 2000
Ametryne	TA	H.	Sulfoxidation	LC-UV	Lang et al. 1997
Atrazine	TA	H.	N-Deisopropylation	LC-UV	Lang et al. 1997
				LC/PDA & LC-MS	Joo et al. 2010
Azinophos methyl	OP	I.	Desulfuration	AChE Inh. LC-UV	Buratti et al. 2002
Bioresmethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
Butachlor	ChAc	H.	N-Dealkoxylation	LC-UV	Coleman et al. 2000
Carbaryl	CA	I.	Aromatic hydroxy-lation Methyl Oxidation	LC-UV	Tang et al. 2002
Carbosulfan	CA	I.	N-S cleavage Sulfoxidation	LC-MS	Abass et al. 2010
Chlorpyrifos	OP	I.	Desulfuration	AChE Inh. LC-UV	Buratti et al. 2002
			Desulfuration Dearylation	LC-UV	Tang et al. 2001; Foxenberg et al. 2007; Mutch and Williams 2006; Croom et al. 2010
DEET		I. R.	Aromatic methyloxidation	LC-UV	Usmani et al. 2002
Diazinon	OP	I.	Desulfuration	AChE Inh. LC-UV	Buratti et al. 2002
			Desulfuration Dearylation	LC-UV	Mutch and Williams 2006; Kappers et al. 2001
Dimethoate	OP	I.	Desulfuration	AChE Inh.	Buratti and Testai 2007
Disulfoton	OP	I.	Sulfoxidation	LC-UV	Usmani et al. 2004b
Diuron	PU	H.	N-Demethylation	LC-MS	Abass et al. 2007c
Endosulfan-α	CCD	I.	Sulfoxidation	LC-UV	Casabar et al. 2006
				GC-ECD	Lee et al. 2006
Imidacloprid	NC	I.	Nitroimine reduction	TLC	Schulz-Jander and Casida 2002
Fenthion	OP	I.	Desulfuration Sulfoxidation	LC-UV	Leoni et al. 2008
Malathion	OP	I.	Desulfuration	AChE Inh.	Buratti et al. 2005
Metachlor	ChAc	H.	N-Dealkoxylation	LC-UV	Coleman et al. 2000
Metalaxyl	AcA	F.	O-Demethylation Lactone formation	LC-MS	Abass et al. 2007b
Methiocarb	OP	I.	Sulfoxidation	LC-UV	Usmani et al. 2004b
Methoxychlor	OC	I.	O-Demethylation	TLC	Stresser and Kupfer

					1998
Parathion	OP	I.	Desulfuration	AChE Inh. LC-UV	Buratti et al. 2002
				AChE Inh.	Sams et al. 2000
			Desulfuration Dearylation	LC-UV	Foxenberg et al. 2007; Mutch and Williams 2006; Mutch et al. 2003; Mutch et al. 1999; Butler and Murray 1997
Phorate	OP	I.	Sulfoxidation	LC-UV	Usmani et al. 2004b
Profenofos	OP	I.	Hydroxypropylation Desthiopropylation	LC-MS	Abass et al. 2007a
Terbutryne	TA	H.	Sulfoxidation	LC-UV	Lang et al. 1997
triadimefon	TriA	F.	t-butyl group metabolism	LC-UV	Barton et al. 2006
λ-Cyhalothrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009

Table 5. Pesticides reported to be metabolized at least in part by human CYP2B6.

6.5 CYP2C8

Pesticide	Chemical class	Type	Metabolic pathway	Detection method	Reference
Ametryne	TA	H.	N-Deisopropylation	LC-UV	Lang et al. 1997
Atrazine	TA	H.	N-Deisopropylation	LC/PDA & LC-MS	Joo et al. 2010
Bifenthrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
Bioresmethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
Carbaryl	CA	I.	Aromatic hydroxy-lation Methyl Oxidation	LC-UV	Tang et al. 2002
Carbosulfan	CA	I.	N-S cleavage	LC-MS	Abass et al. 2010
Chlorpyrifos	OP	I.	Desulfuration Dearylation	LC-UV	Mutch and Williams 2006
cis-Permethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
Cypermethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
Deltamethrin	PY	I.	Oxidative metabolism	LC-MS	Godin et al. 2007
Diazinon	OP	I.	Desulfuration Dearylation	LC-UV	Mutch and Williams 2006
Dimethoate	OP	I.	Desulfuration	AChE Inh.	Buratti and Testai 2007
Diuron	PU	H.	N-Demethylation	LC-MS	Abass et al. 2007c
Esfenvalerate	PY	I.	Oxidative metabolism	LC-MS	Godin et al. 2007
Parathion	OP	I.	Desulfuration Dearylation	LC-UV	Mutch and Williams 2006; Mutch et al. 2003
Resmethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009

S-Bioallethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
τ-Permethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
β-Cyfluthrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
λ-Cyhalothrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009

Table 6. Pesticides reported to be metabolized at least in part by human CYP2C8.

6.6 CYP2C9

Pesticide	Chemical class	Type	Metabolic pathway	Detection method	Reference
Ametryne	TA	H.	N-Deisopropylation Sulfoxidation	LC-UV	Lang et al. 1997
Atrazine	TA	H.	N-Deisopropylation	LC/PDA & LC-MS	Joo et al. 2010
Bifenthrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
Bioresmethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
Carbaryl	CA	I.	Aromatic hydroxy-lation Methyl Oxidation	LC-UV	Tang et al. 2002
Chlorpyrifos	OP	I.	Desulfuration Dearylation	LC-UV	Tang et al. 2001; Croom et al. 2010
cis-Permethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
Cypermethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
Diazinon	OP	I.	Desulfuration Dearylation	LC-UV	Kappers et al. 2001
Dimethoate	OP	I.	Desulfuration	AChE Inh.	Buratti and Testai 2007
Disulfoton	OP	I.	Sulfoxidation	LC-UV	Usmani et al. 2004b
Diuron	PU	H.	N-Demethylation	LC-MS	Abass et al. 2007c
Endosulfan-α	CCD	I.	Sulfoxidation	LC-UV	Casabar et al. 2006
Esfenvalerate	PY	I.	Oxidative metabolism	LC-MS	Godin et al. 2007
Fenthion	OP	I.	Desulfuration Sulfoxidation	LC-UV	Leoni et al. 2008
Imidacloprid	NC	I.	Imidazolidine oxidation	TLC	Schulz-Jander and Casida 2002
Methiocarb	OP	I.	Sulfoxidation	LC-UV	Usmani et al. 2004b
Methoxychlor	OC	I.	O-Demethylation	TLC	Stresser and Kupfer 1998
Parathion	OP	I.	Desulfuration Dearylation	LC-UV	Foxenberg et al. 2007
Phorate	OP	I.	Sulfoxidation	LC-UV	Usmani et al. 2004b
Resmethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
S-Bioallethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
Sulprofos	OP	I.	Sulfoxidation	LC-UV	Usmani et al. 2004b
τ-Permethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009

Tributyltin	OT	B. A.	Dealkylation	GC	Ohhira et al. 2006
Triphenyltin	OT	F.; A.; M.	Dearylation	GC	Ohhira et al. 2006
β-Cyfluthrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
λ-Cyhalothrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009

Table 7. Pesticides reported to be metabolized at least in part by human CYP2C9.

6.7 CYP2C19

Pesticide	Chemical class	Type	Metabolic pathway	Detection method	Reference
Ametryne	TA	H.	N-Deethylation N-Deisopropylation	LC-UV	Lang et al. 1997
Atrazine	TA	H.	N-Deisopropylation N-Deethylation	LC-UV LC-UV	Lang et al. 1997
				LC/PDA & LC-MS	Joo et al. 2010
Azinophos methyl	OP	I.	Desulfuration	AChE Inh. & LC-UV	Buratti et al. 2002
Bifenthrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
Bioresmethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
Carbaryl	CA	I.	Aromatic hydroxy-lation Methyl Oxidation	LC-UV	Tang et al. 2002
Carbofuran	CA	I.	Ring oxidation	LC-UV	Usmani et al. 2004a
Carbosulfan	CA	I.	N-S cleavage Sulfoxidation	LC-MS LC-MS	Abass et al. 2010
Chlorpyrifos	OP	I.	Desulfuration	AChE Inh. & LC-UV	Buratti et al. 2002
			Desulfuration Dearylation	LC-UV	Tang et al. 2001; Foxenberg et al. 2007; Mutch and Williams 2006; Croom et al. 2010
cis-Permethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
Cypermethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
DEET		I. R.	N-Deethylation	LC-UV	Usmani et al. 2002
Deltamethrin	PY	I.	Oxidative metabolism	LC-MS	Godin et al. 2007
Diazinon	OP	I.	Desulfuration	AChE Inh. & LC-UV	Buratti et al. 2002
			Desulfuration Dearylation	LC-UV	Mutch and Williams 2006; Kappers et al. 2001
Dimethoate	OP	I.	Desulfuration	AChE Inh.	Buratti and Testai 2007
Disulfoton	OP	I.	Sulfoxidation	LC-UV	Usmani et al. 2004b
Diuron	PU	H.	N-Demethylation	LC-MS	Abass et al. 2007c

Endosulfan-α	CCD	I.	Sulfoxidation	LC-UV	Casabar et al. 2006
Esfenvalerate	PY	I.	Oxidative metabolism	LC-MS	Godin et al. 2007
Fenthion	OP	I.	Desulfuration Sulfoxidation	LC-UV	Leoni et al. 2008
Fipronil	PP	I.	Sulfoxidation	LC-UV	Tang et al. 2004
Furametpyr	OX	F.	N-Demethylation	TLC NMR & MS	Nagahori et al. 2000
Imidacloprid	NC	I.	oxidation	TLC	Schulz-Jander and Casida 2002
Malathion	OP	I.	Desulfuration	AChE Inh.	Buratti et al. 2005
Methiocarb	OP	I.	Sulfoxidation	LC-UV	Usmani et al. 2004b
Methoxychlor	OC	I.	O-Demethylation bis-O-Demethylation	TLC	Stresser and Kupfer 1998
Myclobutanil	TriA	F.	n-butyl metabolism	LC-UV	Barton et al. 2006
Parathion	OP	I.	Desulfuration Dearylation	LC-UV	Foxenberg et al. 2007; Mutch and Williams 2006; Mutch et al. 2003
Parathion	OP	I.	Desulfuration	AChE Inh. & LC-UV	Buratti et al. 2002
Phorate	OP	I.	Sulfoxidation	LC-UV	Usmani et al. 2004b
Profenofos	OP	I.	Hydroxypropylation Desthiopropylation	LC-MS	Abass et al. 2007a
Resmethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
S-Bioallethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
Sulprofos	OP	I.	Sulfoxidation	LC-UV	Usmani et al. 2004b
Terbuthylazine	TA	H.	N-Deethylation	LC-UV	Lang et al. 1997
τ Permethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
triadimefon	TA	F.	t-butyl metabolism	LC-UV	Barton et al. 2006
Tributyltin	OT	B. A.	Dealkylation	GC	Ohhira et al. 2006
Triphenyltin	OT	F.; A.; M.	Dearylation	GC	Ohhira et al. 2006
β-Cyfluthrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
λ-Cyhalothrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009

Table 8. Pesticides reported to be metabolized at least in part by human CYP2C19.

6.8 CYP2D6

Pesticide	Chemical class	Type	Metabolic pathway	Detection method	Reference
Atrazine	TA	H.	N-Deethylation	LC-UV	Lang et al. 1997
Carbaryl	CA	I.	Aromatic hydroxy-lation Methyl Oxidation	LC-UV	Tang et al. 2002
Chlorpyrifos	OP	I.	Desulfuration Dearylation	LC-UV	Mutch and Williams 2006

			Desulfuration	AChE Inh.	Sams et al. 2000
DEET		I. R.	Aromatic methyl oxidation	LC-UV	Usmani et al. 2002
Diazinon	OP	I.	Desulfuration	AChE Inh.	Sams et al. 2000
			Desulfuration Dearylation	LC-UV	Mutch and Williams 2006; Kappers et al. 2001
Disulfoton	OP	I.	Sulfoxidation	LC-UV	Usmani et al. 2004b
Diuron	PU	H.	N-Demethylation	LC-MS	Abass et al. 2007c
Imidacloprid	NC	I.	Nitroimine reduction	TLC	Schulz-Jander and Casida 2002
Methiocarb	OP	I.	Sulfoxidation	LC-UV	Usmani et al. 2004b
Parathion	OP	I.	Desulfuration	LC-UV	Mutch and Williams 2006; Mutch et al. 2003
				AChE Inh.	Sams et al. 2000
Sulprofos	OP	I.	Sulfoxidation	LC-UV	Usmani et al. 2004b

Table 9. Pesticides reported to be metabolized at least in part by human CYP2D6.

6.9 CYP2E1

Pesticide	Chemical class	Type	Metabolic pathway	Detection method	Reference
Atrazine	TA	H.	N-Deethylation N-Deisopropylation	LC-UV	Lang et al. 1997
			N-Deisopropylation	LC/PDA & LC-MS	Joo et al. 2010
Carbaryl	CA	I.	Aromatic hydroxy-lation Methyl Oxidation	LC-UV	Tang et al. 2002
DEET		I. R.	Aromatic methyl oxidation	LC-UV	Usmani et al. 2002
Diuron	PU	H.	N-Demethylation	LC-MS	Abass et al. 2007c
Imidacloprid	NC	I.	Nitroimine reduction	TLC	Schulz-Jander and Casida 2002
Parathion	OP	I.	Desulfuration Dearylation	LC-UV	Mutch and Williams 2006; Mutch et al. 2003

Table 10. Pesticides reported to be metabolized at least in part by human CYP2E1.

6.10 CYP3A4

Pesticide	Chemical class	Type	Metabolic pathway	Detection method	Reference
Acetachlor	ChAc	H.	N-Dealkoxylation	LC-UV	Coleman et al. 2000
Alachlor	ChAc	H.	N-Dealkoxylation Aliphatic hydroxy-lation	LC-UV	Coleman et al. 2000; Coleman et al. 1999
Ametryne	TA	H.	N-Deethylation N-Deisopropylation Sulfoxidation	LC-UV	Lang et al. 1997
Atrazine	TA	H.	N-Deethylation N-Deisopropylation	LC-UV	Lang et al. 1997
				LC/PDA & LC-MS	Joo et al. 2010
Azinophos methyl	OP	I.	Desulfuration	AChE Inh. & LC-UV	Buratti et al. 2002
Bioresmethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
Butachlor	ChAc	H.	N-Dealkoxylation	LC-UV	Coleman et al. 2000
Carbaryl	CA	I.	Aromatic hydroxy-lation Methyl Oxidation	LC-UV	Tang et al. 2002
Carbofuran	CA	I.	Ring oxidation	LC-UV	Usmani et al. 2004a
Carbosulfan	CA	I.	N-S cleavage Sulfoxidation	LC-MS	Abass et al. 2010
Chlorpyrifos	OP	I.	Desulfuration	AchE Inh. & LC-UV	Buratti et al. 2002; Sams et al. 2000; Buratti et al. 2006
			Desulfuration Dearylation	LC-UV	Tang et al. 2001; Foxenberg et al. 2007; Mutch and Williams 2006; Croom et al. 2010; Dai et al. 2001
cis-Permethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
Cypermethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
DEET		I. R.	N-Deethylation	LC-UV	Usmani et al. 2002
Diazinon	OP	I.	Desulfuration	AchE Inh. & LC-UV	Buratti et al. 2002
				AChE Inh.	Sams et al. 2000
			Desulfuration Dearylation	LC-UV	Mutch and Williams 2006; Kappers et al. 2001
Dimethoate	OP	I.	Desulfuration	AChE Inh.	Buratti and Testai 2007
Diniconazole	CZ	F.	Hydroxylation	LC-MS	Mazur and Kenneke 2008
Disulfoton	OP	I.	Sulfoxidation	LC-UV	Usmani et al. 2004b
Diuron	PU	H.	N-Demethylation	LC-MS	Abass et al. 2007c
Endosulfan-α	CCD	I.	Sulfoxidation	LC-UV	Casabar et al. 2006
				GC-ECD	Lee et al. 2006
Endosulfan-β	CCD	I.	Sulfoxidation	GC-ECD	Lee et al. 2006
Epoxiconazole	CZ	F.	Hydroxylation	LC-MS	Mazur and Kenneke 2008
Fenbuconazole	CZ	F.	Hydroxylation	LC-MS	Mazur and Kenneke 2008
Fenthion	OP	I.	Desulfuration Sulfoxidation	LC-UV	Leoni et al. 2008
			Desulfuration	AChE Inh. & LC-UV	Buratti et al. 2006
Fipronil	PP	I.	Sulfoxidation	LC-UV	Tang et al. 2004

Furametpyr	OX	F.	N-Demethylation	TLC NMR & MS	Nagahori et al. 2000
Hexachlorobenzene	OC	I.	Aromatic hydroxylation	TLC NMR & MS	Mehmood et al. 1996
Hexaconazole	CZ	F.	Hydroxylation	LC-MS	Mazur and Kenneke 2008
Imidacloprid	NC	I.	Imidazolidine oxidation Nitroimine reduction	TLC	Schulz-Jander and Casida 2002
Ipconazole	CZ	F.	Hydroxylation	LC-MS	Mazur and Kenneke 2008
Malathion	OP	I.	Desulfuration	AChE Inh. & LC-UV	Buratti et al. 2005; Buratti et al. 2006
Metalaxyl	AcA	F.	Ring hydroxylation Methyl hydroxylation O-Demethylation Lactone formation	LC-MS	Abass et al. 2007b
Metconazole	CZ	F.	Hydroxylation	LC-MS	Mazur and Kenneke 2008
Methiocarb	OP	I.	Sulfoxidation	LC-UV	Usmani et al. 2004b
Myclobutanil	TA	F.	n-butyl metabolism	LC-UV	Barton et al. 2006
Myclobutanil	TA	F.	Aliphatic hydroxylation	LC-MS	Mazur and Kenneke 2008
Paclobutrazole	TA	PGR	Hydroxylation	LC-MS	Mazur and Kenneke 2008
Parathion	OP	I.	Desulfuration	AChE Inh. & LC-UV	Buratti et al. 2002; Buratti et al. 2006
			Desulfuration	AChE Inh.	Sams et al. 2000
			Desulfuration Dearylation	LC-UV	Foxenberg et al. 2007; Mutch and Williams 2006; Mutch et al. 2003; Mutch et al. 1999; Butler and Murray 1997
Pentachlorobenzene	OC	I.	Aromatic hydroxylation	TLC NMR & MS	Mehmood et al. 1996
Phorate	OP	I.	Sulfoxidation	LC-UV	Usmani et al. 2004b
Profenofos	OP	I.	Hydroxypropylation Desthiopropylation	LC-MS	Abass et al. 2007a
Propiconazole	CZ	F.	Aliphatic hydroxylation	LC-MS	Mazur and Kenneke 2008
Resmethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
S-Bioallethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
Sulprofos	OP	I.	Sulfoxidation	LC-UV	Usmani et al. 2004b
Terbuthylazine	TA	H.	N-Deethylation	LC-UV	Lang et al. 1997
Terbutryne	TA	H.	N-Deethylation Sulfoxidation	LC-UV	Lang et al. 1997
t-Bromuconazole	CZ	F.	Aromatic hydroxylation	LC-MS	Mazur and Kenneke 2008
τ-Permethrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
triadimefon	TA	F.	t-butyl group metabolism	LC-UV	Barton et al. 2006
Tributyltin	OT	BA.	Dealkylation	GC	Ohhira et al. 2006
Triphenyltin	OT	F. A. M.	Dearylation	GC	Ohhira et al. 2006
Triticonazole	CZ	F.	Hydroxylation	LC-MS	Mazur and Kenneke 2008
Uniconazole	CZ	PGR.	Hydroxylation	LC-MS	Mazur and Kenneke 2008
β-Cyfluthrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009
λ-Cyhalothrin	PY	I.	Oxidative metabolism	LC-MS	Scollon et al. 2009

Table 11. Pesticides reported to be metabolized at least in part by human CYP3A4.

6.11 CYP3A5

Pesticide	Chemical class	Type	Metabolic pathway	Detection method	Reference
Carbaryl	CA	I.	Aromatic hydroxy-lation Methyl Oxidation	LC-UV	Tang et al. 2002
Carbosulfan	CA	I.	N-S cleavage Sulfoxidation	LC-MS	Abass et al. 2010
Chlorpyrifos	OP	I.	Desulfuration Dearylation	LC-UV LC-UV	Foxenberg et al. 2007; Mutch and Williams 2006; Croom et al. 2010
			Desulfuration	AChE Inh. & LC-UV	Buratti et al. 2006
DEET		I. R.	N-Deethylation	LC-UV	Usmani et al. 2002
Deltamethrin	PY	I.	Oxidative metabolism	LC-MS	Godin et al. 2007
Diazinon	OP	I.	Desulfuration Dearylation	LC-UV	Mutch and Williams 2006
Diuron	PU	H.	N-Demethylation	LC-MS	Abass et al. 2007c
Endosulfan-α	CCD	I.	Sulfoxidation	GC-ECD	Lee et al. 2006
Endosulfan-β	CCD	I.	Sulfoxidation	GC-ECD	Lee et al. 2006
Esfenvalerate	PY	I.	Oxidative metabolism	LC-MS	Godin et al. 2007
Fenthion	OP	I.	Desulfuration	AChE Inh. & LC-UV	Buratti et al. 2006
Malathion	OP	I.	Desulfuration	AChE Inh. & LC-UV	Buratti et al. 2006
Myclobutanil	TriA	F.	n-butyl metabolism	LC-UV	Barton et al. 2006
Parathion	OP	I.	Desulfuration Dearylation	LC-UV	Foxenberg et al. 2007; Mutch and Williams 2006; Mutch et al. 2003; Mutch et al. 1999
			Desulfuration	AChE Inh. & LC-UV	Buratti et al. 2006
Sulprofos	OP	I.	Sulfoxidation	LC-UV	Usmani et al. 2004b

Table 12. Pesticides reported to be metabolized at least in part by human CYP3A5.

6.12 CYP3A7

Pesticide	Chemical class	Type	Metabolic pathway	Detection method	Reference
Atrazine	TA	H.	N-Deisopropylation	LC/PDA & LC-MS	Joo et al. 2010
Carbosulfan	CA	I.	N-S cleavage Sulfoxidation	LC-MS	Abass et al. 2010
Chlorpyrifos	OP	I.	Desulfuration Dearylation	LC-UV	Foxenberg et al. 2007; Croom et al. 2010
			Desulfuration	AChE Inh. & LC-UV	Buratti et al. 2006
Endosulfan-α	CCD	I.	Sulfoxidation	LC-UV	Casabar et al. 2006
Fenthion	OP	I.	Desulfuration	AChE Inh. & LC-UV	Buratti et al. 2006
Malathion	OP	I.	Desulfuration	AChE Inh. & LC-UV	Buratti et al. 2006
Parathion	OP	I.	Desulfuration Dearylation	LC-UV	Foxenberg et al. 2007
			Desulfuration	AChE Inh. & LC-UV	Buratti et al. 2006

Table 13. Pesticides reported to be metabolized at least in part by human CYP3A7.

6.13 Metabolic reactions

Table 14 contains information classified according to individual metabolic reactions and the corresponding pesticides.

Reactions	Pesticides	CYP enzymes involved at least in part
Aliphatic hydroxylation	Alachlor; myclobutanil; propiconazole	CYP3A4
	Carbaryl	CYP1A1; CYP1A2; CYP3A4
	Hexachlorobenzene; pentachlorobenzene; τ-bromuconazole	CYP3A4
Aromatic methyl oxidation	DEET	CYP2B6
bis-O-Demethylation	Methoxychlor	CYP2C18
Dealkylation	Tributyltin	CYP2C9; CYP2C18; CYP2C19; CYP3A4
Dearylation	Chlorpyrifos; diazinon	CYP1A2; CYP2A6; CYP2B6;CYP2C9; CYP2C19; CYP2D6; CYP3A4; CYP3A5
	Parathion	CYP2C19; CYP3A4; CYP2B6; CYP2C8; CYP3A5; CYP1A2;
	Triphenyltin	CYP2C9; CYP2C18; CYP2C19; CYP3A4

Desthiopropylation	Profenofos	CYP3A4; CYP2B6
Desulfuration	Azinophos methyl	CYP2C19; CYP3A4
	Chlorpyrifos	CYP2C19; CYP3A4; CYP2B6; CYP3A5; CYP2D6; CYP3A7
	Diazinon	CYP1A2; CYP2A6; CYP2B6;CYP2C9; CYP2C19; CYP2D6; CYP3A4; CYP3A5
	Dimethoate	CYP1A2; CYP3A4
	Fenthion; malathion	CYP1A2; CYP2B6; CYP3A4; CYP3A5; CYP3A7
	Parathion	CYP2C19; CYP3A4; CYP2B6; CYP2C8; CYP3A5; CYP2C8; CYP2D6
Hydroxylation	Diniconazole; epoxiconazole; fenbuconazole; hexaconazole; ipconazole; metconazole; paclobutrazole; triticonazole; uniconazole	CYP3A4
Hydroxypropylation	Profenofos	CYP2B6; CYP2C19
Imidazolidine oxidation	Imidacloprid	CYP3A4
Lactone formation	Metalaxyl	CYP2B6
Methyl Oxidation	Carbaryl	CYP1A2; CYP2B6
n-butyl side-chain metabolism	Myclobutanil	CYP2C19
N-Dealkoxylation	Acetachlor; alachlor; butachlor	CYP3A4; CYP2B6
	Metachlor	CYP2B6
N-Deethylation	Ametryn; atrazine; terbuthylazine; terbutryne	CYP1A1 CYP1A2 CYP2C19 CYP3A4
	DEET	CYP2C19
N-Deisopropylation	Ametryne; atrazine	CYP1A1; CYP1A2CYP2B6 CYP2E1 CYP2C8 CYP2C9 CYP2C19 CYP3A4, CYP3A7
N-Demethylation	Diuron	CYP1A1; CYP1A2; CYP2C19; CYP3A4
	Furametpyr	CYP1A2; CYP2C19
Nitroimine reduction	Imidacloprid	CYP3A4
N-S cleavage	Carbosulfan	CYP3A4; CYP3A5
O-Demethylation	Metalaxyl	CYP2B6
	Methoxychlor	CYP1A2; CYP2C19
Oxidative metabolism	Bifenthrin; s-bioallethrin; λ-cyhalothrin	CYP2C19
	Bioresmethrin; cypermethrin; τ -permethrin	CYP1A2; CYP2C19
	cis-permethrin; resmethrin	CYP2C9; CYP2C19
	Deltamethrin	CYP2C8; CYP2C19; CYP3A5
	Esfenvalerate	CYP2C8; CYP2C19; CYP3A5; CYP2C9
	τ-cyfluthrin	CYP2C8; CYP2C19
Ring hydroxylation	Metalaxyl	CYP3A4

Ring oxidation	Carbofuran	CYP3A4
Sulfoxidation	Ametryn	CYP1A2
	Carbosulfan	CYP1A1; CYP2B6; CYP3A5
	Disulfoton; phorate; sulprofos	CYP2C9; CYP2C18; CYP2C19
	Endosulfan-α	CYP2B6; CYP3A4
	Endosulfan-β	CYP3A4; CYP3A5
	Fenthion; methiocarb	CYP2C9; CYP2C19
	Fipronil	CYP3A4
	Terbutryne	CYP1A2; CYP3A4
t-butyl group metabolism	Triadimefon	CYP2C19

Table 14. Type of reactions catalyzed at least in part by CYPs in one or more corresponding pesticide biotransformation.

7. Induction of CYP enzymes

Induction is defined as an increase in enzyme activity associated with an increase in intracellular enzyme concentration. CYP-pesticides interactions involve either induction or inhibition of metabolizing enzymes. Many induction studies have been conducted in vitro using primary human hepatocytes, human hepatoma cell lines or cell lines derived from other human tissues (Dierickx, 1999; Delescluse et al. 2001; Coumoul et al. 2002; Sanderson et al. 2002; Wyde et al. 2003; Lemaire et al. 2004). Primary culture of hepatocyte maintain whole cell metabolism since transporters and both phase I and phase II enzymes are present. Likewise, HepaRG cells express a large panel of liver-specific genes including several CYP enzymes, which is in contrast to HepG2 cell lines. In addition to P450 enzymes, HepaRG cells have a stable expression of phase II enzymes, transporters and nuclear transcription factors over a time period of six weeks in culture (Aninat et al. 2006; Anthérieu et al. 2010; Kanebratt and Andersson, 2008; Turpeinen et al. 2009).

Both immunoblotting and reverse transcription polymerase chain reaction (RT-PCR) techniques have been applied to examine the pesticide-CYP induction (Wyde et al. 2003; Lemaire et al. 2004; Das et al. 2006; Sun et al. 2005; Johri et al. 2007; Barber et al. 2007). However, problems in tissue availability, interindividual differences, reproducibility and ethical issues preclude the efficient large-scale use of human primary hepatocytes for induction screening.

One important factor regulating the expression of drug metabolising enzymes is induction by a diverse group of endogenous and exogenous substances that bind to the nuclear receptors pregnane X receptor (PXR) or constitutive androstane receptor (CAR), thereby causing significant up-regulation of gene transcription (Pelkonen et al. 2008; Handschin and Meyer, 2003). Therefore, the development of mechanism-based test systems for induction screening, based for example on in vitro pregnane X receptor/constitutive androstane receptor activation, is currently very active, and some test systems are in use as a first step for the identification of potential inducers (Pelkonen et al. 2005; Pelkonen and Raunio, 2005).

Whereas the acute effects of exposure to high doses of pesticides are well known, the long-term effects of lower exposure levels remain controversial. The ability of chemicals to induce metabolic enzymes, including cytochrome P450 (CYP), has long been considered as one of

the most sensitive biochemical cellular responses to toxic insult (Gonzalez et al. 1993; Denison and Whitlock Jr., 1995), since it often occurs at much lower doses of the chemical than those known to cause lethal or overtly toxic effects. Assessment of inducibility of xenobiotic-metabolising enzymes by pesticides is vital for health risk assessment. Numerous pesticides are capable of inducing their own metabolism and by enzyme induction can also lead to enhanced biotransformation of other xenobiotics. Several articles on CYP gene inducibility by pesticides and other chemicals used in agriculture and public health have been published (Abass et al. 2009) and a review article dealing with CYP gene modulation by pesticides is needed.

8. Acknowledgements

This work was supported by a grant from KONE foundation - Finland.

9. References

Abass, K.; Reponen, P. & Pelkonen, O. (2009). Metabolic and interactions properties of selected fungicides, In: *Fungicides: chemistry, environmental impact and health effects,* De Costa, P. & Bezerra, P., (Ed.), (25-62), Nova Science Publisher, ISBN: 978-1-60692-631-4, New York

Abass, K.; Reponen, P.; Jalonen, J. & Pelkonen, O. (2007a). In vitro metabolism and interaction of profenofos by human, mouse and rat liver preparations. *Pestic. Biochem. Physiol.*, Vol. 87, No. 3, (238-247).

Abass, K.; Reponen, P.; Jalonen, J. & Pelkonen, O. (2007b). In vitro metabolism and interactions of the fungicide metalaxyl in human liver preparations. *Environ. Toxicol. Pharmacol.*, Vol. 23, No. 1, (39-47).

Abass, K.; Reponen, P.; Turpeinen, M.; Jalonen, J. & Pelkonen, O. (2007c). Characterization of diuron N-demethylation by mammalian hepatic microsomes and cDNA-expressed human cytochrome P450 enzymes. *Drug Metab. Dispos.*, Vol. 35, No. 9, (1634-1641).

Abass, K.; Reponen, P.; Mattila, S. & Pelkonen, O. (2010). Metabolism of carbosulfan II. Human interindividual variability in its in vitro hepatic biotransformation and the identification of the cytochrome P450 isoforms involved. *Chem. Biol. Interact.*, Vol. 185, No. 3, (163-173).

Adams, D.R.; Jones, A.M.; Plopper, C.G.; Serabjit-Singh, C.J. & Philpot, R.M. (1991). Distribution of cytochrome P-450 monoxygenase enzymes in the nasal mucosa of hamster and rat. *Am. J. Anat.*, Vol. 190, No. 3, (291-298).

Ahokas, J. & Pelkonen, O. (2007). Pharmacokinetics: How Does The Body Handle Drugs? In: *Pharmacology, Encyclopedia of Life Support Systems (EOLSS),* UNESCO, (Ed.), UNESCO Publishing-Eolss Publishers, , Oxford, UK

Aninat, C.; Piton, A.; Glaise, D.; Le Charpentier, T.; Langouet, S.; Morel, F.; Guguen-Guillouzo, C. & Guillouzo, A. (2006). Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells. *Drug Metab Dispos*, Vol. 34, No. 1, (75-83).

Anthérieu, S.; Chesné, C.; Li, R.; Camus, S.; Lahoz, A.; Picazo, L.; Turpeinen, M.; Tolonen, A.; Uusitalo, J.; Guguen-Guillouzo, C. & Guillouzo, A. (2010). Stable expression, activity, and inducibility of cytochromes P450 in differentiated HepaRG cells. *Drug Metab. Dispos.*, Vol. 38, No. 3, (516-525).

Barber, D.S.; McNally, A.J.; Garcia-Reyero, N. & Denslow, N.D. (2007). Exposure to p,p'-DDE or dieldrin during the reproductive season alters hepatic CYP expression in largemouth bass (Micropterus salmoides). *Aquatic Toxicology*, Vol. 81, No. 1, (27-35).

Barton, H.A.; Tang, J.; Sey, Y.M.; Stanko, J.P.; Murrell, R.N.; Rockett, J.C. & Dix, D.J. (2006). Metabolism of myclobutanil and triadimefon by human and rat cytochrome P450 enzymes and liver microsomes. *Xenobiotica*, Vol. 36, No. 9, (793-806).

Beaune, P.H.; Kremers, P.G.; Kaminsky, L.S.; De Graeve, J.; Albert, A. & Guengerich, F.P. (1986). Comparison of monooxygenase activities and cytochrome P-450 isozyme concentrations in human liver microsomes. *Drug Metab. Dispos.*, Vol. 14, No. 4, (437-442).

Bergh, A.F. & Strobel, H.W. (1992). Reconstitution of the brain mixed function oxidase system: Purification of NADPH-cytochrome P450 reductase and partial purification of cytochrome P450 from whole rat brain. *J. Neurochem.*, Vol. 59, No. 2, (575-581).

Berthou, F.; Dreano, Y.; Belloc, C.; Kangas, L.; Gautier, J. & Beaune, P. (1994). Involvement of cytochrome P450 3A enzyme family in the major metabolic pathways of toremifene in human liver microsomes. *Biochem. Pharmacol.*, Vol. 47, No. 10, (1883-1895).

Bourrie, M.; Meunier, V.; Berger, Y. & Fabre, G. (1996). Cytochrome P450 isoform inhibitors as a tool for the investigation of metabolic reactions catalyzed by human liver microsomes. *J. Pharmacol. Exp. Ther.*, Vol. 277, No. 1, (321-332).

Brandon, E.F.A.; Raap, C.D.; Meijerman, I.; Beijnen, J.H. & Schellens, J.H.M. (2003). An update on in vitro test methods in human hepatic drug biotransformation research: pros and cons. *Toxicol. Appl. Pharmacol.*, Vol. 189, No. 3, (233-246).

Buratti, F.M.; Volpe, M.T.; Fabrizi, L.; Meneguz, A.; Vittozzi, L. & Testai, E. (2002). Kinetic parameters of OPT pesticide desulfuration by c-DNA expressed human CYPs. *Environ. Toxicol. Pharmacol.*, Vol. 11, No. 3-4, (181-190).

Buratti, F.M.; D'Aniello, A.; Volpe, M.T.; Meneguz, A. & Testai, E. (2005). Malathion bioactivation in the human liver liver: The contribution of different cytochrome P450 isoform. *Drug Metab. Dispos.*, Vol. 33, No. 3, (295-302).

Buratti, F.M.; Leoni, C. & Testai, E. (2006). Foetal and adult human CYP3A isoforms in the bioactivation of organophosphorothionate insecticides. *Toxicol. Lett.*, Vol. 167, No. 3, (245-255).

Buratti, F.M. & Testai, E. (2007). Evidences for CYP3A4 autoactivation in the desulfuration of dimethoate by the human liver. *Toxicology*, Vol. 241, No. 1-2, (33-46).

Butler, A.M. & Murray, M. (1997). Biotransformation of parathion in human liver: Participation of CYP3A4 and its inactivation during microsomal parathion oxidation. *J. Pharmacol. Exp. Ther*, Vol. 280, No. 2, (966-973).

Casabar, R.C.T.; Wallace, A.D.; Hodgson, E. & Rose, R.L. (2006). Metabolism of endosulfan-{alpha} by human liver microsomes and its utility as a simultaneous in vitro probe for CYP2B6 and CYP3A4. *Drug Metab. Dispos.*, Vol. 34, No. 10, (1779-1785).

Clarke, S.E.; Ayrton, A.D. & Chenery, R.J. (1994). Characterization of the inhibition of P4501A2 by furafylline. *Xenobiotica*, Vol. 24, No. 6, (517).

Coleman, S.; Liu, S.; Linderman, R.; Hodgson, E. & Rose, R.L. (1999). In vitro metabolism of alachlor by human liver microsomes and human cytochrome P450 isoforms. *Chem. Biol. Interact.*, Vol. 122, No. 1, (27-39).

Coleman, S.; Linderman, R.; Hodgson, E. & Rose, R.L. (2000). Comparative metabolism of chloroacetamide herbicides and selected metabolites in human and rat liver microsomes. *Environ. Health Perspect.*, Vol. 108, No. 12, (1151).

Coumoul, X.; Diry, M. & Barouki, R. (2002). PXR-dependent induction of human CYP3A4 gene expression by organochlorine pesticides. *Biochemical Pharmacology*, Vol. 64, No. 10, (1513-1519).

Croom, E.L.; Wallace, A.D. & Hodgson, E. (2010). Human variation in CYP-specific chlorpyrifos metabolism. *Toxicology*, Vol. 276, No. 3, (184-191).

Dai, D.; Tang, J.; Rose, R.; Hodgson, E.; Bienstock, R.J.; Mohrenweiser, H.W. & Goldstein, J.A. (2001). Identification of variants of CYP3A4 and characterization of their abilities to metabolize testosterone and chlorpyrifos. *J. Pharmacol. Exp. Ther.*, Vol. 299, No. 3, (825-831).

Das, G.P.; Shaik, A.P. & Jamil, K. (2006). Cytotoxicity and genotoxicity induced by the pesticide profenofos on cultured human peripheral blood lymphocytes. *Drug Chem. Toxicol.*, Vol. 29, No. 3, (313).

Delescluse, C.; Ledirac, N.; Li, R.; Piechocki, M.P.; Hines, R.N.; Gidrol, X. & Rahmani, R. (2001). Induction of cytochrome P450 1A1 gene expression, oxidative stress, and genotoxicity by carbaryl and thiabendazole in transfected human HepG2 and lymphoblastoid cells. *Biochemical Pharmacology*, Vol. 61, No. 4, (399-407).

Denison, M.S. & Whitlock Jr., J.P. (1995). Xenobiotic-inducible transcription of cytochrome P450 genes. *J. Biol. Chem.*, Vol. 270, No. 31, (18175-18178).

Dhawan, A.; Parmar, D.; Das, M. & Seth, P.K. (1990). Cytochrome P-450 dependent monooxygenases in neuronal and glial cells: Inducibility and specificity. *Biochem. Biophys. Res. Commun.*, Vol. 170, No. 2, (441-447).

Dierickx, P.J. (1999). CYP1/2 activation and glutathione-dependent cytotoxicity of four pesticides in Hep G2 and Fa32 cells. *Toxicology in Vitro*, Vol. 13, No. 4-5, (779-783).

Dutcher, J.S. & Boyd, M.R. (1979). Species and strain differences in target organ alkylation and toxicity by 4-ipomeanol predictive value of covalent binding in studies of target organ toxicities by reactive metabolites. *Biochemical Pharmacology*, Vol. 28, No. 23, (3367-3372).

Ekins, S.; Mäenpää, J. & Wrighton, S.A. (1999). In vitro metabolism: subcellular fractions, In: *Handbook of drug metabolism,* Woolf, T.F., (Ed.), (369-399), Marcel Dekker, , New York

Ekins, S.; Ring, B.J.; Grace, J.; McRobie-Belle, D.J. & Wrighton, S.A. (2000). Present and future in vitro approaches for drug metabolism. *J. Pharmacol. Toxicol. Methods*, Vol. 44, No. 1, (313-324).

Eriksson, C. & Brittebo, E.B. (1991). Metabolic activation of the herbicide dichlobenil in the olfactory mucosa of mice and rats. *Chem. Biol. Interact.*, Vol. 79, No. 2, (165-177).

Foxenberg, R.J.; McGarrigle, B.P.; Knaak, J.B.; Kostyniak, P.J. & Olson, J.R. (2007). Human hepatic cytochrome P450-specific metabolism of parathion and chlorpyrifos. *Drug Metab. Dispos.*, Vol. 35, No. 2, (189-193).

Godin, S.J.; Crow, J.A.; Scollon, E.J.; Hughes, M.F.; DeVito, M.J. & Ross, M.K. (2007). Identification of rat and human cytochrome P450 isoforms and a rat serum esterase that metabolize the pyrethroid insecticides deltamethrin and esfenvalerate. *Drug Metab. Dispos.*, Vol. 35, No. 9, (1664-1671).

Gonzalez, F.J.; Liu, S. & Yano, M. (1993). Regulation of cytochrome P450 genes: molecular mechanisms. *Pharmacogenetics and Genomics*, Vol. 3, No. 1, (51-57).

Guengerich, F.P. (1995). Cytochromes P450 of human liver. Classification and activity profiles of the major enzymes, In: *Advances in drug metabolism in man,* Pacifici, G.M. & Fracchia, G.N., (Ed.), (179-231), Office for the Official Publications of the European Communities, ISBN: 978-9282739822, Luxembourg

Guengerich, F.P. (1999). Cytochrome P-450 3A4: regulation and role in drug metabolism. *Annu. Rev. Pharmacol. Toxicol.*, Vol. 39, No. 1, (1-17).

Guengerich, F.P. (1995). Cytochromes P450 of human liver. Classification and activity profiles of the major enzymes, In: *Advances in drug metabolism in man. Office for the Official Publications of the European Communities,* Pacifici, G.M. & Fracchia, G.N., (Ed.), (179-231), , Luxembourg

Guengerich, F.P. (2001). Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. *Chem. Res. Toxicol.*, Vol. 14, No. 6, (611-650).

Handschin, C. & Meyer, U.A. (2003). Induction of Drug Metabolism: The Role of Nuclear Receptors. *Pharmacological Reviews*, Vol. 55, No. 4, (649-673).

Hjelle, J.; Hazelton, G.; Klaassen, C. & Hjelle, J. (1986). Glucuronidation and sulfation in rabbit kidney. *J. Pharmacol. Exp. Ther.*, Vol. 236, No. 1, (150-156).

Hodgson, E. & Goldstein, J.A. (2001). Metabolism of toxicants: phase I reactions and pharmacogenetics, In: *Introduction to Biochemical Toxicology,* Hodgson, E. & Smart, R.C., (Ed.), (67-113), Wiely, New York

Hodgson, E. (2001). In vitro human phase I metabolism of xenobiotics I: Pesticides and related chemicals used in agriculture and public health. *J Biochem Mol Toxicol,* Vol.15 (296–299)

Hodgson, E. (2003). In vitro human phase I metabolism of xenobiotics I: Pesticides and related compounds used in agriculture and public health, may 2003. *J. Biochem. Mol. Toxicol.*, Vol. 17, No. 4, (201-206).

Jacolot, F.; Simon, I.; Dreano, Y.; Beaune, P.; Riche, C. & Berthou, F. (1991). Identification of the cytochrome P450 IIIA family as the enzymes involved in the N-demethylation of tamoxifen in human liver microsomes. *Biochem. Pharmacol.*, Vol. 41, No. 12, (1911-1919).

Johri, A.; Yadav, S.; Dhawan, A. & Parmar, D. (2007). Overexpression of cerebral and hepatic cytochrome P450s alters behavioral activity of rat offspring following prenatal exposure to lindane. *Toxicology and Applied Pharmacology*, Vol. 225, No. 3, (278-292).

Joo, H.; Choi, K. & Hodgson, E. (2010). Human metabolism of atrazine. *Pestic. Biochem. Physiol.*, Vol. 98, No. 1, (73-79).

Kanebratt, K.P. & Andersson, T.B. (2008). Evaluation of HepaRG cells as an in vitro model for human drug metabolism studies. *Drug Metab. Dispos.*, Vol. 36, No. 7, (1444-1452).

Kappers, W.A.; Edwards, R.J.; Murray, S. & Boobis, A.R. (2001). Diazinon Is Activated by CYP2C19 in Human Liver. *Toxicol. Appl. Pharmacol.*, Vol. 177, No. 1, (68-76).

Khan, W.A.; Park, S.S.; Gelboin, H.V.; Bickers, D.R. & Mukhtar, H. (1989). Monoclonal antibodies directed characterization of epidermal and hepatic cytochrome P-450 isozymes induced by skin application of therapeutic crude coal tar. *J. Invest. Dermatol.*, Vol. 93, No. 1, (40-45).

Lang, D.H.; Rettie, A.E. & Bocker, R.H. (1997). Identification of enzymes involved in the metabolism of atrazine, terbuthylazine, ametryne, and terbutryne in human liver microsomes. *Chem. Res. Toxicol.*, Vol. 10, No. 9, (1037-1044).

Lawton, M.; Gasser, R.; Tynes, R.; Hodgson, E. & Philpot, R. (1990). The flavin-containing monooxygenase enzymes expressed in rabbit liver and lung are products of related but distinctly different genes. *J. Biol. Chem.*, Vol. 265, No. 10, (5855-5861).

LeBlanc, G.A. & Dauterman, W.C. (2001). Conjugation and elimination of toxicants, In: *Introduction to Biochemical Toxicology,* Hodgson, E. & Smart, R.C., (Ed.), Wiley, ISBN: 9780838543320, New York

Lee, H.; Moon, J.; Chang, C.; Choi, H.; Park, H.; Park, B.; Lee, H.; Hwang, E.; Lee, Y.; Liu, K. & Kim, J. (2006). Stereoselective metabolism of endosulfan by human liver microsomes and human cytochrome P450 isoforms. *Drug Metab. Dispos.*, Vol. 34, No. 7, (1090-1095).

Lemaire, G.; de Sousa, G. & Rahmani, R. (2004). A PXR reporter gene assay in a stable cell culture system: CYP3A4 and CYP2B6 induction by pesticides. *Biochem. Pharmacol.*, Vol. 68, No. 12, (2347-2358).

Leoni, C.; Buratti, F.M. & Testai, E. (2008). The participation of human hepatic P450 isoforms, flavin-containing monooxygenases and aldehyde oxidase in the biotransformation of the insecticide fenthion. *Toxicol. Appl. Pharmacol.*, Vol. 233, No. 2, (343-352).

Liska, D.; Lyon, M. & Jones, D.S. (2006). Detoxification and biotransformational imbalances. *J Sci Heal*, Vol. 2, No. 2, (122-140).

Low, L.K. (1998). Metabolic changes of drugs and related organic compounds, In: *Wilson and Gisvold's textbook of organic medicinal and pharmaceutical chemistry,* Delgado, J.N. & Remers, W.A., (Ed.), (43–122), Lippincott-Raven, ISBN: 0397515839, Philadelphia

Mazur, C.S. & Kenneke, J.F. (2008). Cross-species comparison of conazole fungicide metabolites using rat and rainbow trout (Onchorhynchus mykiss) hepatic microsomes and purified human CYP 3A4. *Environ. Sci. Technol.*, Vol. 42, No. 3, (947-954).

Mehmood, Z.; Williamson, M.P.; Kelly, D.E. & Kelly, S.L. (1996). Metabolism of organochlorine pesticides: The role of human cytochrome P450 3A4. *Chemosphere*, Vol. 33, No. 4, (759-769).

Mutch, E.; Daly, A.K.; Leathart, J.B.; Blain, P.G. & Williams, F.M. (2003). Do multiple cytochrome P450 isoforms contribute to parathion metabolism in man? *Arch. Toxicol.*, Vol. 77, No. 6, (313-320).

Mutch, E.; Blain, P.G. & Williams, F.M. (1999). The role of metabolism in determining susceptibility to parathion toxicity in man. *Toxicol. Lett.*, Vol. 107, No. 1-3, (177-187).

Mutch, E. & Williams, F.M. (2006). Diazinon, chlorpyrifos and parathion are metabolised by multiple cytochromes P450 in human liver. *Toxicology*, Vol. 224, No. 1-2, (22-32).

Nagahori, H.; Yoshino, H.; Tomigahara, Y.; Isobe, N.; Kaneko, H. & Nakatsuka, I. (2000). Metabolism of furametpyr. 1. identification of metabolites and in vitro biotransformation in rats and humans. *J. Agric. Food Chem.*, Vol. 48, No. 11, (5754-5759).

Nebert, D.W. & Russell, D.W. (2002). Clinical importance of the cytochromes P450. *Lancet*, Vol. 360, No. 9340, (1155-1162).

Oesch, F.; Herrero, M.E.; Hengstler, J.G.; Lohmann, M. & Arand, M. (2000). Metabolic Detoxification: Implications for Thresholds. *Toxicol Pathol*, Vol. 28, No. 3, (382-387).

Ohhira, S.; Enomoto, M. & Matsui, H. (2006). In vitro metabolism of tributyltin and triphenyltin by human cytochrome P-450 isoforms. *Toxicology*, Vol. 228, No. 2-3, (171-177).

Parkinson, A. (2001). Biotransformation of xenobiotics, In: *Casarett and Doull's toxicology : the basic science of poisons,* Klaassen, C.D., (Ed.), (113–186), McGraw-Hill Medical Pub. Division, ISBN: 0071124535 : ¹44.99; 0071347216 (U.S.), New York ; London

Pearce, R.E.; McIntyre, C.J.; Madan, A.; Sanzgiri, U.; Draper, A.J.; Bullock, P.L.; Cook, D.C.; Burton, L.A.; Latham, J.; Nevins, C. & Parkinson, A. (1996). Effects of freezing, thawing, and storing human liver microsomes on cytochrome P450 activity. *Arch. Biochem. Biophys.*, Vol. 331, No. 2, (145-169).

Pelkonen, O.; Turpeinen, M.; Hakkola, J.; Honkakoski, P.; Hukkanen, J. & Raunio, H. (2008). Inhibition and induction of human cytochrome P450 enzymes: current status. *Arch. Toxicol.*, Vol. 82, No. 10, (667-715).

Pelkonen, O.; Kaltiala, E.H.; Larmi, T.K.I. & Karki, N.T. (1974). Cytochrome P 450 linked monooxygenase system and drug induced spectral interactions in human liver microsomes. *Chem. Biol. Interact.*, Vol. 9, No. 3, (205-216).

Pelkonen, O. & Turpeinen, M. (2007). In vitro–in vivo extrapolation of hepatic clearance: Biological tools, scaling factors, model assumptions and correct concentrations. *Xenobiotica*, Vol. 37, No. 10, (1066-1089).

Pelkonen, O. & Raunio, H. (2005). In vitro screening of drug metabolism during drug development: can we trust the predictions? *Expert Opin. Drug Metab. Toxicol.*, Vol. 1, No. 1, (49-59).

Pelkonen, O.; Turpeinen, M.; Uusitalo, J.; Rautio, A. & Raunio, H. (2005). Prediction of drug metabolism and interactions on the basis of in vitro investigations. *Basic Clin. Pharmacol. Toxicol.*, Vol. 96, No. 3, (167-175).

Peters, W.H.M. & Kremers, P.G. (1989). Cytochromes P-450 in the intestinal mucosa of man. *Biochemical Pharmacology*, Vol. 38, No. 9, (1535-1538).

Rendic, S. & Di Carlo, F.J. (1997). Human cytochrome P450 enzymes: A status report summarizing their reactions, substrates, inducers, and inhibitors. *Drug Metab. Rev.*, Vol. 29, No. 1-2, (413-580).

Rodrigues, A.D. (1999). Integrated cytochrome P450 reaction phenotyping. Attempting to bridge the gap between cDNA-expressed cytochromes P450 and native human liver microsomes. *Biochem. Pharmacol.*, Vol. 57, No. 5, (465-480).

Rose, R.L. & Hodgson, E. (2004). Metabolism of toxicants, In: *Text Book of Modern Toxicology,* Hodgson, E., (Ed.), (111-148), Wiley, ISBN: 978-0-470-46206-5, New York

Rostami-Hodjegan, A. & Tucker, G.T. (2007). Simulation and prediction of in vivo drug metabolism in human populations from in vitro data. *Nat. Rev. Drug Discov.*, Vol. 6, No. February, (140-148).

Sams, C.; Mason, H.J. & Rawbone, R. (2000). Evidence for the activation of organophosphate pesticides by cytochromes P450 3A4 and 2D6 in human liver microsomes. *Toxicol. Lett.*, Vol. 116, No. 3, (217-221).

Sanderson, J.T.; Boerma, J.; Lansbergen, G.W.A. & van den Berg, M. (2002). Induction and Inhibition of Aromatase (CYP19) Activity by Various Classes of Pesticides in H295R Human Adrenocortical Carcinoma Cells. *Toxicology and Applied Pharmacology*, Vol. 182, No. 1, (44-54).

Schmider, J.; Greenblatt, D.; von Moltke, L.; Harmatz, J. & Shader, R. (1995). N-demethylation of amitriptyline in vitro: role of cytochrome P-450 3A (CYP3A) isoforms and effect of metabolic inhibitors. *J. Pharmacol. Exp. Ther.*, Vol. 275, No. 2, (592-597).

Schulz-Jander, D.A. & Casida, J.E. (2002). Imidacloprid insecticide metabolism: human cytochrome P450 isozymes differ in selectivity for imidazolidine oxidation versus nitroimine reduction. *Toxicol. Lett.*, Vol. 132, No. 1, (65-70).

Scollon, E.J.; Starr, J.M.; Godin, S.J.; DeVito, M.J. & Hughes, M.F. (2009). In vitro metabolism of pyrethroid pesticides by rat and human hepatic microsomes and cytochrome P450 isoforms. *Drug Metab. Dispos.*, Vol. 37, No. 1, (221-228).

Sesardic, D.; Boobis, A.R.; Murray, B.P.; Murray, S.; Segura, J.; de la Torre, R. & Davis, D.S. (1990). Furafylline is a potent and selective inhibitor of cytochrome P4501A2 in man. *Br. J. Clin. Pharmacol.*, Vol. 29, No. 5, (651-663).

Sohl, C.D.; Isin, E.M.; Eoff, R.L.; Marsch, G.A.; Stec, D.F. & Guengerich, F.P. (2008). Cooperativity in Oxidation Reactions Catalyzed by Cytochrome P450 1A2. *Journal of Biological Chemistry*, Vol. 283, No. 11, (7293-7308).

Stresser, D.M. & Kupfer, D. (1998). Human cytochrome P450-catalyzed conversion of the proestrogenic pesticide methoxychlor into an estrogen. Role of CYP2C19 and CYP1A2 in O-demethylation. *Drug Metab. Dispos.*, Vol. 26, No. 9, (868-874).

Sun, G.; Thai, S.; Tully, D.B.; Lambert, G.R.; Goetz, A.K.; Wolf, D.C.; Dix, D.J. & Nesnow, S. (2005). Propiconazole-induced cytochrome P450 gene expression and enzymatic activities in rat and mouse liver. *Toxicol. Lett.*, Vol. 155, No. 2, (277-287).

Tang, J.; Cao, Y.; Rose, R.L.; Brimfield, A.A.; Dai, D.; Goldstein, J.A. & Hodgson, E. (2001). Metabolism of chlorpyrifos by human cytochrome P450 isoforms and human, mouse, and rat liver microsomes. *Drug Metab. Dispos.*, Vol. 29, No. 9, (1201-1204).

Tang, J.; Cao, Y.; Rose, R.L. & Hodgson, E. (2002). In vitro metabolism of carbaryl by human cytochrome P450 and its inhibition by chlorpyrifos. *Chem. Biol. interact.*, Vol. 141, No. 3, (229-241).

Tang, J.; Tang, J.; Amin Usmani, K.; Hodgson, E. & Rose, R.L. (2004). In vitro metabolism of fipronil by human and rat cytochrome P450 and its interactions with testosterone and diazepam. *Chem. Biol. Interact.*, Vol. 147, No. 3, (319-329).

Testa, B. & Krämer, S. (2006). The Biochemistry of Drug Metabolism ? An Introduction. *Chemistry & Biodiversity*, Vol. 3, No. 10, (1053-1101).

Tolonen, A.; Turpeinen, M.; Uusitalo, J. & Pelkonen, O. (2005). A simple method for differentiation of monoisotopic drug metabolites with hydrogen–deuterium exchange liquid chromatography/electrospray mass spectrometry. *European Journal of Pharmaceutical Sciences*, Vol. 25, No. 1, (155-162).

Tolonen, A.; Petsalo, A.; Turpeinen, M.; Uusitalo, J. & Pelkonen, O. (2007). In vitro interaction cocktail assay for nine major cytochrome P450 enzymes with 13 probe reactions and a single LC/MSMS run: analytical validation and testing with monoclonal anti-CYP antibodies. *J. Mass Spec.*, Vol. 42, No. 7, (960-966).

Tremaine, L.M.; Diamond, G.L. & Quebbemann, A.J. (1985). Quantitative determination of organ contribution to excretory metabolism. *Journal of Pharmacological Methods*, Vol. 13, No. 1, (9-35).

Turpeinen, M.; Uusitalo, J.; Jalonen, J. & Pelkonen, O. (2005). Multiple P450 substrates in a single run: rapid and comprehensive in vitro interaction assay. *Eur. J. Pharm. Sci.*, Vol. 24, No. 1, (123-132).

Turpeinen, M.; Korhonen, L.E.; Tolonen, A.; Uusitalo, J.; Juvonen, R.; Raunio, H. & Pelkonen, O. (2006). Cytochrome P450 (CYP) inhibition screening: Comparison of three tests. *Eur. J. Pharm. Sci.*, Vol. 29, No. 2, (130-138).

Turpeinen, M.; Tolonen, A.; Chesne, C.; Guillouzo, A.; Uusitalo, J. & Pelkonen, O. (2009). Functional expression, inhibition and induction of CYP enzymes in HepaRG cells. *Toxicol In Vitro*, Vol. 23, No. 4, (748-753).

Usmani, K.A.; Rose, R.L.; Goldstein, J.A.; Taylor, W.G.; Brimfield, A.A. & Hodgson, E. (2002). In vitro human metabolism and interactions of repellent N,N-diethyl-m-toluamide. *Drug Metab. Dispos.*, Vol. 30, No. 3, (289-294).

Usmani, K.A.; Hodgson, E. & Rose, R.L. (2004a). In vitro metabolism of carbofuran by human, mouse, and rat cytochrome P450 and interactions with chlorpyrifos, testosterone, and estradiol. *Chem. Biol. Interact.*, Vol. 150, No. 3, (221-332).

Usmani, K.A.; Karoly, E.D.; Hodgson, E. & Rose, R.L. (2004b). In vitro sulfoxidation of thioether compounds by human cytochrome P450 and flavin-containing monooxygenase isoforms with particular reference to the CYP2C subfamily. *Drug Metab. Dispos.*, Vol. 32, No. 3, (333-339).

White, R.E. (2000). High-throughput screening in drug metabolism and pharmacokinetic support of drug discovery. *Annu. Rev. Pharmacol. Toxicol.*, Vol. 40, No. 1, (133-157).

Wolkers, J.; Witkamp, R.F.; Nijmeijer, S.M.; Burkow, I.C.; de Groene, E.M.; Lydersen, C.; Dahle, S. & Monshouwer, M. (1998). Phase I and phase II enzyme activities in Ringed seals (Phoca hispida): characterization of hepatic cytochrome P450 by activity patterns, inhibition studies, mRNA analyses, and western blotting. *Aquatic Toxicol.*, Vol. 44, No. 1-2, (103-115).

Wyde, M.E.; Bartolucci, E.; Ueda, A.; Zhang, H.; Yan, B.; Negishi, M. & You, L. (2003). The Environmental Pollutant 1,1-Dichloro-2,2-bis (p-chlorophenyl)ethylene Induces Rat

Hepatic Cytochrome P450 2B and 3A Expression through the Constitutive Androstane Receptor and Pregnane X Receptor. *Mol Pharmacol*, Vol. 64, No. 2, (474-481).

Yamazaki, H.; Inoue, K.; Turvy, C.G.; Guengerich, F.P. & Shimada, T. (1997). Effects of freezing, thawing, and storage of human liver samples on the microsomal contents and activities of cytochrome P450 enzymes. *Drug Metab. Dispos.*, Vol. 25, No. 2, (168-174).

Zamek-Gliszczynski, M.J.; Hoffmaster, K.A.; Nezasa, K.; Tallman, M.N. & Brouwer, K.L.R. (2006). Integration of hepatic drug transporters and phase II metabolizing enzymes: mechanisms of hepatic excretion of sulfate, glucuronide, and glutathione metabolites. *Eur. J. Pharm. Sci.*, Vol. 27, No. 5, (447-486).

Zanger, U.; Turpeinen, M.; Klein, K. & Schwab, M. (2008). Functional pharmacogenetics/ genomics of human cytochromes P450 involved in drug biotransformation. *Anal. Bioanal. Chem.*, Vol. 392, No. 6, (1093-1108).

9

Insect Management with Aerosols in Food-Processing Facilities

Dhana Raj Boina and Bhadriraju Subramanyam
Department of Grain Science and Industry
Kansas State University, Manhattan, KS
USA

1. Introduction

Stored-product insects infest raw grain, processed cereal grains, warehouses, and food-processing facilities such as flour and feed mills. The presence of insects in commodities or structures leads to quantitative and qualitative losses of grain and processed food. The presence of insects and insect-related materials (insect fragments) in processed products is regulated by federal food and drug laws. Management of insects in food-processing facilities is important to prevent adulteration of finished products. Several tactics that are recommended for management of insects associated with food-processing facilities include stock rotation, inspection of inbound and outbound materials, sanitation, exclusion practices, fumigation with sulfuryl fluoride or methyl bromide, use of heat treatments (Mahroof et al. 2003, Boina et al. 2008), crack and crevice treatments with residual insecticides (Toews et al. 2005, 2009), and the use of aerosols or fogs. A lot of research has been done documenting the effectiveness of whole structure treatments with fumigants and heat treatments, and limited information is available on efficacy of the other chemical and non-chemical (sanitation) insect management tactics. Among these, application of insecticides as aerosols (fogging) is one tool with great potential for effective management of insects in food-processing facilities based on pilot-scale and a few commercial-scale tests. In this chapter, we provide a detailed account on the history of aerosol technology and suitability of this technology for insect management in food-storage and food-processing facilities with supporting information from studies conducted in laboratory and field settings with notes on advantages and limitations of this technology.

2. Brief history of aerosols and stored product insect management

In a typical aerosol application for food-processing facilities, the pressurized insecticide formulation is dispensed through a specialized hand-held, portable or preinstalled applicator's nozzles with fine openings to deliver spray particles of 5-50 μm in size as a mist or fog into a confined space (Peckman and Arthur 2006). These applications are also known as fogging, ultra-low volume (ULV) or space treatments depending on the type of formulation used and particle sizes dispersed. The concept is to have the aerosol particles settle on exposed flying or crawling insects and poison them with little or significant residual activity to insects coming into contact with settled particles. The application of

insecticides as aerosols for controlling stored-product insects is an old technique and was initially used for controlling tobacco pests such as the cigarette beetle, *Lasioderma serricorne* (F.), in tobacco warehouses (Tenhet et al. 1957, 1958). The first and the most widely used insecticide for such application was an organophosphate, dichlorvos (DDVP) (Tenhet et al. 1957, 1958; Gillenwater and Harein 1964, Jay et al. 1964, Press and Childs 1966, Childs et al. 1966, Childs 1967, Harein et al. 1970, 1971). In the initial experiments, in contrast to our perception of aerosol treatment where insect control is achieved by deposition of aerosol particles on exposed insects, the efficacy of dichlorvos against *L. serricorne* life stages was measured by exposing them to the dichlorvos vapor (which permeated 30 meters of air space from the point of release) by introducing life stages of insects into the treated warehouse 2 h after the aerosol particles had settled down to the floor (Tenhet et al 1958). Based on the findings from this and other studies, the commercial application of dichlorvos aerosol at a daily application rate of 0.5 g AI/28.3 m^3 headspace was begun in tobacco warehouses for controlling *L. serricorne* and the tobacco moth, *Ephestia elutella* (Hübner).

In 1960s and 1970s, another group of researchers started working on the use of dichlorvos aerosol for controlling insects other than those associated with stored tobacco (Gillenwater and Harein 1964, Jay et al. 1964, Harein et al. 1970, 1971; Gillenwater et al. 1971; Cogburn and Simonaitis 1975). In the late 1970s, some researchers evaluated various synthetic pyrethroids and organophosphates as aerosols for controlling stored-product insects in transport vehicles such as tractor trailers and transport trailer vans (Kirkpatrick and Gillenwater 1979, 1981; Halliday et al. 1987). Then in late 1980s and early 1990s, some studies focused on evaluating synergized pyrethroids as alternatives to dichlorvos for controlling stored-product insects in laboratory settings in artificially constructed air-tight chambers (Arthur 1988, 1993; Arthur and Gillenwater 1990). The current trend in aerosol applications is to evaluate efficacy of neurotoxic aerosols such as dichlorvos, synergized pyrethrins, and pyrethroids alone or combined with insect growth regulators (IGRs) such as methoprene, hydroprene, and pyriproxyfen under laboratory and field conditions (air-tight chambers or flour mills, warehouses and other food-processing facilities) applied via portable application devices or permanently installed systems within the facilities (Jenson et al. 2010a; Phillips and Throne 2010).

From the beginning of this technology in late 1950s until now, several types of application devices have been used for delivering aerosol insecticides for controlling stored-product insects. These include pressurized cylinder-based automatic aerosol dispensing systems (Childs et al. 1966) and pressurized cans which are held in hand and a person, with proper personal protective apparel, walks through the middle (aisle) of a storage facility or a transport vehicle releasing the aerosol (Kirkpatrick and Gillenwater 1979, 1981). Alternatively, a person stands outside the facility and releases the aerosol by directing the aerosol can inside the facility through a window or door (Arthur 1988, 1993; Arthur and Gillenwater 1990). Some researchers used resin pellets impregnated with an insecticide, mainly dichlorvos, for releasing insecticide in vapor form either by a dispenser for providing large quantities of vapor (Gillenwater and Harein 1964, Harein et al. 1971) or by placing pellets in a wire mesh tray to release the vapor into a confined space of a facility (Gillenwater et al. 1971). In some studies, researchers have used portable devices or equipment to release the insecticide aerosols into the facilities such as a DeVilbiss sprayer equipped with an air-atomizing nozzle (Jay et al. 1964), a 'Tifa'generating thermal aerosol

machine (Childs 1967), a Dyna-Fog 70 thermal aerosol machine, a 6-nozzle McGill Fog-Trol pneumatic aerosol machine, a rotary-whip ULV applicator (Cogburn and Simonaitis 1975), a Micro-Gen S1W-5E unit dispenser (Bernhard and Bennett 1981), and a hand-held ULV applicator (Jenson et al. 2010b). Some of the devices mentioned above such as rotary-whip ULV applicator or vapor dispenser have a built-in fan or blower for dispersing the aerosol particles more efficiently within a facility (Gillenwater and Harein 1964, Harein et al. 1971, Cogburn and Simonaitis 1975).

All the aerosol application devices described above are either hand-held or portable and can be moved within and between facilities. Nevertheless, the growing need for repeated applications of aerosols for controlling insects in food-processing facilities necessitated the installation of a permanent overhead automatic dispensing system (Childs 1967) or aerosol/ULV compressed air application systems either to the roof or some other permanent structures in the middle of a warehouse/facility (Arthur 2008, Arthur and Campbell 2008, Arthur 2010, Jenson et al. 2010a). The installation of such permanent aerosol dispensing systems reduces the aerosol treatment costs in the long run. Freon 11/12 in 50:50 ratio and carbon dioxide (CO_2) are the two most common propellants used in aerosol formulations of insecticides available as pressurized cans or cylinders for use with permanently installed dispensing systems, because these propellants aid in aerosol dispersion when released.

Although use of a non-synergized insecticide such as dichlorvos was very common when aerosol technology was introduced, pyrethrins and pyrethroids, used as aerosols later, contained a synergist, piperonyl butoxide, for improving the efficacy of aerosols against stored-product insects (Bernhard and Bennett 1981, Arthur 1988, 1993, Arthur and Gillenwater 1990). The latest strategy in aerosol technology is to use a combination of insecticides. The most widely evaluated treatment in both laboratory and field settings is combining a synergized pyrethrin or pyrethroid with an IGR (Arthur 2008, 2010; Jenson et al. 2010a,b; Sutton et al. 2011), because the synergized pyrethrins or pyrethroids are effective against all life stages of insect pest species and provide quick knockdown but fail to provide longer periods of insect control due to poor residual activity. The IGRs are effective against immature life stages of pest species and provide longer periods of protection due to their slow degradation on deposited surfaces compared to synergized pyrethrins or pyrethorids (Jenson et al. 2010a, Phillips and Throne 2010). Table 1 presents the list of insecticides used and/or available for application as aerosols for controlling insects in food-storage and food-processing facilities.

3. Distribution and efficacy of aerosol insecticides in laboratory settings

Several food-storage and food-processing facilities do not permit researchers or scientists to bring live insects into the facilities for conducting experiments. Therefore, it is common practice to conduct these experiments under laboratory conditions in artificially constructed cardboard boxes, wooden chambers, or sheet metal chambers to simulate field conditions (Harein et al. 1971, Arthur 1988, 1993; Arthur and Gillenwater 1990, Jenson et al. 2010a). Several studies were conducted under laboratory settings to determine the distribution/dispersion pattern of aerosol insecticides in the facility by assessing the mortality of important stored-product insects placed in the experimental chamber.

For successful management of stored-product insects in facilities, uniform distribution of applied aerosol throughout the facility, including underneath the wooden pallets used for

storage of raw or finished food products and inside pieces of equipment, where the insects take refuge is of utmost importance. Jenson et al. (2010a) studied the effect of a synthetic pyrethroid, esfenvalerate, and an IGR methoprene, applied as aerosols either alone at the label rate or combined at full or reduced label rates and distributed uniformly throughout an artificially constructed small wooden sheds on the mortality of eggs and larvae of the Indianmeal moth, *Plodia interpunctella* (Hübner). The insect stages were exposed in Petri dishes placed at various locations in the sheds. Some locations were open (aerosol particle deposition was not obstructed in anyway) and some were concealed (aerosol deposition was obstructed in a greater way as Petri dishes were placed inside equipment or under a wooden pallet). The application rate (full or reduced label rates), application type (alone or combined), and location of dishes with insects influenced the efficacy of aerosol treatment against the target insect life stages (Jenson et al. 2010a). Therefore, it may be a good idea to evaluate the efficacy of candidate aerosols against insect pests under laboratory settings first to determine and understand factors influencing efficacy under field conditions.

Application of aerosols at regular intervals not only controls the insects in warehouses and storage facilities that are flying but also prevents the movement of insects from infested to clean product packages either within a same stack or between stacks stored in a room. For instance, Harein et al. (1971) demonstrated that weekly application of dichlorvos as a vapor at a concentration of 3-4 µg/L volume of air effectively prevented the spread of adults of the confused flour beetle, *Tribolium confusum* (Jacquelin du Val), lesser grain borer, *Rhyzopertha dominica* (L.), and *L. serricorne* from infested to uninfested stacked flour for 5 months in air-tight chambers. Dichlorvos is a fast degrading insecticide with poor residual activity because chemical analysis of packaged flour collected in the treated chamber showed negligible amounts of dichlorvos residues (<0.1 ppm) indicating that dichlorvos is a safer insecticide to use as an aerosol for controlling insects in warehouses with finished packaged foods as it leaves minimal residues (Harein et al. 1971).

The success of an aerosol treatment in controlling insects in food-storage and food-processing facilities depends on the level of harborage available to the insect and duration of exposure of insects to aerosol particles. In a laboratory study, Bernhard and Bennett (1981) showed that the harborage level and exposure time to aerosol play a role in degree of insect control achieved in facilities. Simulating various levels of insect harborage by completely opening to completely closing the cabinet doors containing insects in an air-tight chamber, they showed that adults of the rice weevil, *Sitophilus oryzae* (L.), were more susceptible than adults of *T. confusum* to a synergized pyrethrin applied as an ULV with particle sizes ranging from 5.7-28.6 µm. The adult mortality was none to negligible at maximum harborage level and it increased as the harborage level decreased giving a maximum mortality at no harborage level. Similarly, the adult mortality increased with increase in exposure time from 15 to 120 minutes. Bernhard and Bennett (1981) concluded that aerosols applied at ULV with 5.7-28.6 µm particle size ranges would be most effective only during application and the first 15 minutes after application, because larger droplets carrying the most of active ingredient settle down in the initial 15 minutes and the effectiveness of treatment decreases drastically thereafter. The above conclusion was supported by less than 10% mortality of adults of *T. confusum* and *S. oryzae* obtained when they were introduced in the treatment room 15 or 60 minutes after the application of synergized pyrethrins (Bernhard and Bennett 1981).

In an effort to find safer alternative aerosols to dichlorvos for managing insects in food-storage and food-processing facilities, Arthur (1988, 1993) and Arthur and Gillenwater (1990) conducted several experiments in artificially constructed air-tight chambers (42.5 m^3). These studies evaluated the distribution and efficacy of three pyrethroids, prallethrin, esfenvalerate, and cyfluthrin with or without a synergist at various rates and exposure times against three moth and six beetle species. The results from these studies showed that three moth species [*P. interpunctella*, the almond moth, *Cadra cautella* (Walker), and *E. elutella*] were highly susceptible to pyrethroids tested compared to the beetle species. This conclusion was based on the fact that irrespective of the formulation, exposure time and presence or absence of synergist, near complete to complete knockdown and subsequent mortality of moths was obtained even at the lowest rates tested (Arthur 1988, 1993; Arthur and Gillenwater 1990). Although a near complete to complete knockdown of adults of all beetle species was noticed for all the treatments, most of them recovered and the final mortality ranged from 10-90% depending on the type of pyrethroid (synergized or non-synergized), formulation, rate, and exposure time (Arthur 1988, 1993; Arthur and Gillenwater 1990). The inherent higher susceptibility of moths to pyrethroids than beetles combined with release of moths in the chamber freely, which improved the chances of moths coming into contact with descending aerosol particles than that of beetles (confined to Petri dishes), resulted in greater mortality of moth species than beetle species. Furthermore, within beetle species, the susceptibility to an aerosol insecticide was species-specific because the relative susceptibility of two closely related insect species varied with the aerosol treatment. For instance, *T. confusum* was more sensitive to synergized esfenvalerate than *T. castaneum*, while *T. castaneum* was more sensitive to synergized prallethrin than *T. confusum* (Arthur and Gillenwater 1990, Arthur 1993).

In a few laboratory studies, it was observed that insects that survived the first application of aerosol were completely killed when the second application of the same aerosol was made within the next 1, 2, or 3 days or a week. This can be attributed to the stress from the previous exposure (Arthur and Gillenwater 1990; Arthur 1993). For example, adults of *T. confusum* and *T. castaneum* that survived the first application of prallethrin and esfenvalerate aerosols, respectively, were killed after exposure to a second application made at same the rate after 1, 2, or 3 days (Arthur and Gillenwater 1990; Arthur 1993). A similar cumulative effect of an aerosol insecticide (dichlorvos) against stored-product insects was also reported by Attfield and Webster (1966). Additionally, the post-exposure time between knockdown and complete adult mortality was reduced as the interval between the first and second application increased. In other words, adults died quickly when second application was made 3 days after the first versus 1 day after the first application (Arthur 1993). This finding with prallethrin and esfenvalerate suggests that a second application of these aerosols at least within 7-10 days following the first application will lead to complete control of *T. confusum* and *T. castaneum* adults (Arthur and Gillenwater 1990; Arthur 1993). However, these findings need to be confirmed with tests conducted under field conditions. Also, retaining the insects in the same exposed dishes instead of transferring them to new dishes may have resulted in increased mortality of exposed insects (Arthur and Gillenwater 1990).

4. Distribution and efficacy of aerosol insecticides in field settings

In practical commercial facilities, when the space treatments with aerosol insecticides are made, the deposition of the dispersed aerosol particles on the floor may be obstructed by the

presence of several barriers such as walls, equipment, as well as processed or raw food material that are either shelved or stacked on wooden pallets. Therefore, the influence of barriers within a facility or structure on distribution pattern of dispersed aerosol particles and the consequent effect on insect lethality needs to be evaluated. Gauging the effectiveness of an aerosol treatment based on mortality/survival of resident insect populations in a facility is difficult because of our inability to determine total number of insect species, insect life stages, and numbers present. Commercial food-baited and sticky traps with pheromone lures have been used to gauge effectiveness of treatment intervention (Roesli et al. 2003), but these commercial traps capture only the adult stages, and it is difficult from trap captures to determine whether the insects in traps are resident populations or those that immigrated into the facility from outside.

In the initial experiments conducted using dichlorvos as the aerosol, the life stages of *L. serricorne* were exposed to the vapors but not to the dispersed aerosol particles. The exposure of insect life stages to vapors was achieved by introducing the insects 2 h after aerosol application in wire-mesh cages suspended above the floor surface by which the aerosol particles might have settled down on the floor (Tenhet et al. 1958). In this way, exposure of older eggs, young larvae, and adults of *L. serricorne* in wire-mesh cages to dichlorvos vapors for 1 h in tobacco warehouses resulted in complete mortality. Based on these findings, application of dichlorvos formulation weekly twice at 2 g/28.3 m^3 headspace was recommended for controlling *L. serricorne* in tobacco warehouses (Tenhet et al. 1958). On the other hand, the tobacco moth *E. elutella* control required less dosage because weekly applications of dichlorvos aerosol at 1 g AI/28.3 m^3 headspace reduced populations of this species in tobacco storage warehouses in Virginia, North Carolina, and South Carolina by 99% in 1960 over 1959 and 90% in 1961 over 1960 based on trap catches (Press and Childs 1966). However, Childs et al. (1966) reported that a higher dose than that recommended previously was required for controlling *L. serricorne* adults in tobacco warehouses as daily application of dichlorvos at 0.5 g/28.3 m^3 headspace via an automatic dispensing system in a tobacco warehouse effectively controlled adults based on reduced trap captures.

In late 1970s, efforts made to control *L. serricorne* adults in tobacco warehouses by combining dichlorvos aerosol with hydrogen cyanide (HCN) fumigation yielded good results. Of the various combinations tested, annual fumigation with HCN at 1360.8 g/28.3 m^3 headspace and dichlorvos applied daily at 0.5 g/28.3 m^3 headspace 10 days after fumigation completely controlled *L. serricorne* adults in tobacco warehouses in Virginia, North Carolina, and South Carolina containing flue-cured tobacco solely based on adult trap catch data. Furthermore, it was noticed that warehouses with flue-cured tobacco treated with this combination did not need a second fumigation with HCN for controlling *L. serricorne* for at least two years (Childs 1967).

In all the field studies mentioned above, the effectiveness of an aerosol treatment was gauged based on trap catch data. Although the effectiveness of an aerosol treatment can be gauged to some extent with the number of insects caught in sticky, pheromone or pitfall traps over time, this method is not completely reliable as traps can collect insects that may have immigrated into the facility after the treatment (Arbogast et al. 2000, 2002, 2005, Campbell et al. 2002, 2003, 2004; Roesli et al. 2003, Toews et al. 2006). Therefore, it is a common practice to confine the laboratory-reared insects in wire-mesh cages or Petri dishes and place them across the treatment room or facility at open, obstructed and concealed

locations such as underneath equipment and stacked raw or processed material as well as within equipment. In addition to exposing insects alone, some studies included flour as a food source in the Petri dishes either during or after the exposure to aerosol. Therefore, the findings reported below from various field studies conducted in mills, warehouses and other facilities were based on the exposure of Petri dishes with or without insects and/or flour during an aerosol application.

Findings from a field study conducted in three port warehouses using three different aerosol application devices indicated that the level of insect control obtained in warehouses is influenced by the type of device used and circulation of air inside the food-processing facility during and after the application (Cogburn and Simonaitis 1975). The application of dichlorvos aerosol through pneumatic application device (still air) resulted in the lowest mortality of all three test species (adults of *T. confusum* and *L. serricorne* and larvae of *C. cautella*); moderate mortality was obtained when applied with a thermal aerosol (still air) and the highest mortality [*T. castaneum* (98-99%), *L. serricorne* (97-99%) and *C. cautella* (86-94%)] was obtained when the treatment was supplemented with air circulation via external fans (thermal aerosol) or built-in fan (rotary-whip ULV application device). The uniform mortality of all three test species obtained in wire-mesh cages placed across each warehouse including at concealed locations with air circulation versus uneven mortality (0 to 100%) at various locations without air circulation is a clear indication that circulation of air during aerosol treatment may improve the overall treatment effectiveness. This is mainly attributed to efficient distribution and deposition of aerosol particles into inaccessible areas such as equipment and wooden pallets and shelves via air circulation which in turn helped in killing the insects harbored in these areas. Application of aerosols in commercial facilities is done after the air handling system is shut down and any vents sealed, in order to maintain lethal concentration of particles suspended in air. The presence of air currents results in an exponential decay of the particles in air.

During aerosol applications, the deposition of aerosol particles on open surfaces is achieved primarily by their vertical downward movement, the speed of which is based on particle diameter. However, in obstructed and concealed places such as inside equipment and underneath wooden pallets the deposition is primarily achieved by the tendency of particles to move horizontally (drift) but at a lesser magnitude. Because of this, several studies reported more or less uniform distribution of aerosol particles throughout the facility measured by mortality of test insects placed at various locations across the facility (Arthur 2008, 2010; Jenson et al. 2010a,b). The uniform distribution of aerosol particles of esfenvalerate + methoprene under laboratory settings (Jenson et al. 2010a) and synergized pyrethrin alone and in combination with methoprene or pyriproxyfen under field conditions in a flour mill or warehouse (Arthur 2008, 2010; Jenson et al. 2010b) can be attributed to the above phenomenon. In these studies, irrespective of the location of the aerosol deposit collected such as open and obstructed/concealed in the mill, similar level of mortality of target insect life stages was achieved either due to direct exposure to aerosol particles or deposited residues. This could be due to the deposit being well above that required to cause complete mortality of insects.

Field studies conducted in a flour mill or warehouse suggested that use of synergized pyrethrins (1 or 3%), in combination with methoprene, is more effective (synergistic effect) than using either one alone for controlling eggs of *P. interpunctella* (Jenson et al. 2010b) or

larvae of *T. castaneum* and *T. confusum* (Sutton et al. 2011). The findings also suggested that a higher suppression of adult emergence from eggs can be obtained when unexposed eggs were added to the diets exposed to the above aerosol combination than direct exposure of eggs without diet, perhaps due to consumption of or contact with aerosol-treated diet. Similarly, greater reduction in emergence of adults from 4-wk-old larvae of *T. castaneum* and *T. confusum* was obtained when larvae were added to the flour or various food-packaging materials exposed to the above aerosol combination in concrete poured Petri dishes (Sutton et al. 2011). Combining synergized pyrethrins with methoprene or pyriproxyfen (juvenile hormone mimic) resulted in longer residual activity against immatures of *T. castaneum* and *T. confusum* (Arthur 2010, Sutton et al. 2011). For instance, the 0- to 16-wk-old residues of the synergized pyrethrin + methoprene collected in concrete poured Petri dishes with flour or various food packaging materials during an aerosol application in a mill environment effectively controlled the immature life stages of *T. castaneum* and *T. confusum* that were added later to the Petri dishes. The residual activity of synergized pyrethrin + pyriproxyfen was shorter than the above combination as only few adults emerged from eggs and larvae of *T. castaneum* and larvae of *T. confusum* exposed to the residues that were 0- to 10-wk-old. Increasing the concentration of pyrethrin only (from 1 to 3%) in the combination treatment has proportionately increased the residual activity against eggs and larvae but not pupae of *T. castaneum.* The combined application of synergized pyrethrin and methoprene not only resulted in increased residual activity but also caused various morphological deformities in larvae of *T. castaneum* and *T. confusum* (Sutton et al. 2011). The longer residual activity of above aerosol combinations could be the result of quick knockdown and mortality effects imparted by synergized pyrethrins in the early days and slow and prolonged toxic effect imparted by the IGR in subsequent days (Mondal and Parween 2000, Phillips and Throne 2010, Sutton et al. 2011).

Although synergized pyrethrins when combined with IGRs did not show any effect on adults of *T. confusum* and *T. castaneum* (Arthur 2010), synergized pyrethrins applied alone as an aerosol at the label rate in a large storage room of a commercial food bank effectively controlled the larvae, pupae, and adults of *T. confusum* and *T. castaneum* exposed in Petri dishes even in the presence of flour as a food source at open locations, and the mortality increased with an increase in the post-exposure time (Arthur 2008). Similarly, methoprene applied alone at the label rate was highly toxic to larvae of *T. confusum* (open locations) and *T. castaneum* (open and concealed locations) but moderately toxic to eggs of *P. interpunctella* exposed in media (Arthur 2008, Jenson et al. 2010b).

Gillenwater et al. (1971), Arthur and Campbell (2008), and Toews et al. (2010) showed that both exposure location and presence of flour as the food source in a facility influenced insect control. Synergized pyrethrins applied at the label rate in an empty warehouse resulted in a wide variation in mortality (20-94%) of *T. confusum* adults exposed in Petri dishes without flour in the rear part of the warehouse whereas more than 80% mortality occurred in Petri dishes that were placed in the front part of the warehouse (Arthur and Campbell 2008). This discrepancy was partly explained by the fact that the nozzles delivering aerosol particles were directed to the front of the experimental room (Arthur and Campbell 2008), suggesting non-uniform distribution throughout the empty warehouse. Toews et al. (2010) observed that exposure to synergized pyrethrin and esfenvalerate applied separately as aerosols at label rates resulted in higher mortality of all life stages of *T. castaneum* in open locations than in concealed locations (under wooden pallets).

The exposure of *T. confusum* adults to synergized pyrethrins in Petri dishes with flour in open locations caused complete adult knockdown. However, the knocked down adults recovered later. The recovery and survival were positively correlated with post-exposure time and the amount of flour present in dishes (Arthur and Campbell 2008). Similar observations of knockdown and recovery of *T. castaneum* life stages exposed to synergized pyrethrins and esfenvalerate applied separately at label rates in pilot scale warehouses were made in the presence of flour as food, both in open and concealed locations (under wooden pallets) (Toews et al. 2010). Arthur and Campbell (2008) noticed that *T. confusum* adults exposed to the synergized pyrithrins in Petri dishes with flour during application and later transferred to new Petri dishes along with the same flour showed increased survival. This finding is similar to that observed in laboratory studies with esfenvalerate against *T. castaneum* adults exposed in Petri dishes without flour (Arthur and Gillenwater 1990). The plausible reasons may be related to absorption of aerosol by the flour, resulting in sub-lethal exposures.

The recovery and survival of *T. confusum* and *T. castaneum* life stages observed in the presence of flour as food in the above studies and with contact residual insecticides such as diatomaceous earth and cyfluthrin (Arthur 2000a,b) emphasizes the need for sanitation of facilities before aerosol applications. Furthermore, the fact that a 10% insect survival (90 percent control or 1-log reduction in pest numbers) in a food-processing facility after an insect control operation is sufficient to reach the population to its original density within a month (equivalent to one generation cycle at optimum temperatures) (Hagstrum and Flinn 1992), mainly due to increased oviposition rate by surviving females with abundant food and less competition (Campbell and Runnion 2003). This finding also stresses the need for sanitation of a facility prior to aerosol treatment. With proper sanitation the proportion of insects directly exposed to aerosol during application is considerably increased either by preventing them from taking refuge in the flour patches or forcing them to come out of their refugia. On the other hand, sanitation reduces the chances of insects coming into contact with food either during or after an aerosol application. As no or poor sanitation undermines the actual efficacy of aerosol insecticides, proper sanitation is important for realizing the maximum effectiveness of aerosol treatments in controlling insects in food-processing facilities.

Kirkpatrick and Gillenwater (1979) and Halliday et al. (1987) conducted experiments to control stored-product insects in tractor trailers with several synthetic pyrethroid insecticides. The exposure of adults of *T. confusum* and larvae of the black carpet beetle, *Attagenus megatoma* Lateille (*A. unicolor* Brahm) and larger cabinet beetle, *Trogoderma inclusum* LeConte, to various formulations of *cis*-Permethrin, permethrin and *d*-Phenothrin applied at 5 g of formulation /28.3 m^3 headspace occurred for 30 minutes in Petri dishes (without food). After exposure, insects were transferred to clean Petri dishes. Varying levels of knockdown of insects were observed but most of the *T. confusum* adults recovered and a wide variation in the recovery was observed with other two species depending upon the exposure location and formulation type. Based on these results Kirkpatrick and Gillenwater (1979) and Halliday et al. (1987) concluded that higher rates of aerosol formulations per unit volume of space and longer exposure times are required for controlling insects in cargo vehicles to meet quarantine treatment standards. Despite the increase in application rate (5 to 10 g) and exposure time (30 minutes to 10 h), there was no significant difference in terms

of percent knockdown and percent mortality of the test species (Kirkpatrick and Gillenwater 1979, 1981). This showed that control of insects in transport vehicles is different from warehouses and food-processing facilities and requires an entirely different set of aerosol formulations and treatment protocols.

5. Suitability and adoption of aerosol technology for present pest problem situations in food-processing facilities

The ban on use of ozone-depleting fumigant, methyl bromide, for insect control in food-processing facilities (Fields and White 2002, Anonymous 2004) and problems associated with other fumigants such as development of resistance in insects, corrosion of exposed electrical and metal surfaces, development of fumigation management plans (phosphine) and need for higher doses to kill some insect life stages and reduced effectiveness against embryonic stages of insects (sulfuryl fluoride) (Bond et al. 1984, Arthur et al. 1988, Halliday et al. 1988, Zettler 1990) make aerosols a promising and viable tool for management of insect pests in food-storage and food-processing facilities. This technology is gaining popularity because of its low cost and the ability to do tactical treatments. The feasibility of treating only a portion of a facility makes aerosol application a desirable option for facility managers.

The insecticides applied as aerosols mainly belong to three classes with different modes of action namely organophosphates, pyrethrins/pyrethroids and IGRs (Table 1). Aerosol insecticides belonging to organophosphate and pyrethrins/pyrethroid classes such as dichlorvos, synergized pyrethrins, esfenvalerate, and cyfluthrin are toxic to all life stages of stored-product insects with short residual activity, while IGRs such as methoprene, pyriproxyfen, and hydroprene are toxic to immatures of all stored-product insects (as they cause morphologic and gonadotropic effects) with long residual activity, with some exceptions.

Except for a few earlier studies, a majority of the studies conducted in warehouses and mills proved that insecticides applied as aerosols using portable application devices or permanently installed aerosol application systems distributed the particles uniformly throughout the facility including underneath the wooden pallets (with stacked finished food) and inside equipment based on the similar level of insect control obtained in Petri dishes or wire-mesh cages placed at various locations. Our ongoing studies also proved that dichlorvos aerosol particles distributed uniformly throughout the facility and were able to penetrate into inaccessible areas through horizontal movement and killed insects that were placed in concealed locations. However, the rapid dissipation of residues significantly reduced the overall residual activity of applied dichlorvos. For instance, in a recent unpublished study conducted in a pilot flour mill at Kansas State University, dichlorvos applied at labeled rate killed most of the adults of *T. confusum* (as only few adults survived in concealed locations) exposed in concrete poured Petri dishes placed at various locations classified as open, obstructed and concealed in the first floor of the mill after 24 h exposure to the aerosol (Table 2). The results did not significantly change with additional 24 h holding of exposed insects to the residues on concrete disks in the same Petri dishes because complete mortality in open and obstructed locations and near complete mortality in concealed locations was already achieved during the first 24 h (Table 2). Petri dishes with aerosol deposits after 24 h were brought to the laboratory and exposed to adults of *T.*

Class	Insecticide	Mode of action	Reference
Organophosphate	Dichlorvos (DDVP)	Acetylcholinesterase inhibitor	Childs (1967) Cogburn and Simonaitis (1975) Gillenwater et al. (1971) Harein et al. (1971)
Natural pyrethrins	Synergized pyrethrins	Sodium ion channel modulator	Bernhard and Bennett (1981) Jenson et al. (2010b) Arthur and Campbell (2008) Toews et al. (2010)
Synthetic pyrethroids	cis-Permethrin	Sodium ion channel modulator	Kirkpatrick and Gillenwater (1979) Halliday et al. (1987)
	Permethrin	Sodium ion channel modulator	Kirkpatrick and Gillenwater (1979) Halliday et al. (1987)
	d-Phenothrin	Sodium ion channel modulator	Kirkpatrick and Gillenwater (1979)
	Phenothrin	Sodium ion channel modulator	Halliday et al. (1987)
	Synergized prallethrin	Sodium ion channel modulator	Arthur 1993, 2008
	Esfenvalerate	Sodium ion channel modulator	Jenson et al. (2010a) Toews et al. (2010)
	Fenvalerate	Sodium ion channel modulator	Halliday et al. (1987)
	Cypermethrin	Sodium ion channel modulator	Halliday et al. (1987)
	Fluvalinate	Sodium ion channel modulator	Halliday et al. (1987)
	Cyfluthrin	Sodium ion channel modulator	Halliday et al. (1987)
	Fenpropathrin	Sodium ion channel modulator	Halliday et al. (1987)
	Cyphenothrin	Sodium ion channel modulator	Halliday et al. (1987)
	Synergized resmethrin	Sodium ion channel modulator	Halliday et al. (1987)
	Synergized esfenvalerate	Sodium ion channel modulator	Arthur and Gillenwater (1990)
	Synergized cyfluthrin	Sodium ion channel modulator	Arthur (1988)
Insect growth regulator	Methoprene	Juvenile hormone analog	Arthur (2008) Jenson et al. (2010a)
	Pyriproxyfen	Juvenile hormone mimic	Arthur (2010)
Combinations	Dichlorvos + Pyrethrins	Acetylcholinesterase inhibitor Sodium ion channel modulator	Toews et al. (2006)
	Synergized pyrethrins + Methoprene	Sodium ion channel modulator Juvenile hormone analog	Arthur (2010) Jenson et al. (2010b)
	Esfenvalerate + Methoprene	Sodium ion channel modulator Juvenile hormone analog	Sutton et al. (2011) Jenson et al. (2010a)
	Synergized pyrethrin + Pyriproxyfen	Sodium ion channel modulator Juvenile hormone mimic	Arthur (2010)

Table 1. Insecticides used and/or available for aerosol application for managing stored-product insects.

Treatment	Mean (±SE) percent survival			
	Control[a]	Open	Obstructed[b]	Concealed[c]
Exposure during application (24 h)	100.0 ± 0.0a	0.0 ± 0.0b	0.0 ± 0.0b	6.7 ± 6.7b
Exposure during application (24 h) + 24 h on residues	97.2 ± 2.8a	0.0 ± 0.0b	0.0 ± 0.0b	10.0 ± 10.0b
Exposure to 0 h old residues	97.2 ± 2.8a	66.7 ± 8.8a	53.3 ± 26.7a	86.7 ± 8.8a
Exposure to 24 h old residues	97.2 ± 2.8a	96.7 ± 3.3a	100.0 ± 0.0a	96.7 ± 3.3a

[a]Petri dishes were placed in a laboratory growth chamber at 28°C and 65% RH.
[b]Petri dishes were placed below pieces of equipment or other barriers.
[c]Petri dishes were placed inside pieces of equipment.
Means (n = 3) among treatments for each row (control, open, obstructed, or concealed) followed by different letters are significantly different from one another (least significant difference test, $P < 0.05$).

Table 2. Survival of *T. confusum* adults exposed in concrete poured Petri dishes that were placed in different locations to dichlorvos aerosol applied in a flour mill.

confusum. A majority of these adults survived suggesting rapid dissipation of residues after 24 h. Holding the dishes for an additional 24 h resulted in greater survival of the exposed adults (Table 2).

6. Advantages and limitations of using aerosols for insect management in food-storage and food-processing facilities

There are several advantages of using aerosol technology for insect control in food-storage and food-processing facilities. The cost of using aerosol insecticides is less compared to fumigation with methyl bromide and sulfuryl fluoride or to heat treatment. For example, the costs based on volume treated in Kansas State University pilot flour mill of 9628 m^3 volume during 2009-2010 for methyl bromide, sulfuryl fluoride, heat treatment based on an average of three separate treatments was US$ 1.76, 3.77, and 3.14/m^3, respectively. In contrast, treatment with the aerosol formulations of esfenvalerate and methoprene each applied alone was US$ 0.0025/m^3 and applied together was US $0.0061/$m^3$ (Jensen, 2010a). Relatively air-tight sealing and documentation to comply with federal regulations are essential when using fumigants, but such requirements are little less stringent when using aerosols. However, air-tight sealing of the facility during aerosol treatments may be necessary if the facility is located in a residential area. Aerosol treatments can be conducted in a portion or the whole facility, and the treatment times are very short (2-4 hours) depending on the product. In the United States, aerosol treatments of food-storage and food-processing facilities are generally made during major holidays or on weekends when the facility is not in operation. Treatments with the fumigants, methyl bromide and sulfuryl fluoride, require a minimum exposure time of 24 hours. For heat treatments, the time may be as short as 24 hours or as long as 34 hours. Like fumigants, aerosol treatments require a period of clearing, which with certain aerosols, could range from 2 to 12 hours (overnight). After an aerosol treatment is conducted, concentrations of certain aerosols need to be monitored to make sure that it is safe for workers to reenter facilities. Nevertheless, the duration for which the facility should be out of operation (shutdown) for an aerosol treatment is much shorter (≤12 h) than that required for fumigation or heat treatments (24-34 h).

The presence of flour as a food source seems to influence effectiveness of aerosol treatments. Some studies showed mortality of *T. confusum* and *T. castaneum* life stages to be unaffected by the presence of flour (Arthur 2008), whereas other studies showed presence of flour either during or after aerosol exposure to increase insect survival (Arthur and Campbell 2008, Toews et al. 2010). Irrespective of these findings, it is always recommended that sanitation of the facility be conducted before an aerosol treatment. This measure forces the insects hiding or taking shelter in flour refugia to come out and increases their chances of exposure to aerosol particles. Aerosol applications can be integrated with other management tactics for controlling insects in food-storage and food-processing facilities such as fumigation, application of residual contact insecticides, and sanitation (Toews et al. 2006).

The main limitation is insecticides applied as aerosols lack the ability to penetrate packaged food. Therefore, insects, mostly in the egg stage, inside packaged food escape the exposure and need to be controlled by fumigation. This limitation can be offset to some extent by doing aerosol treatments in empty warehouses and bringing clean raw or finished or packaged products into the facilities so it reduces the chance of cross contamination and infestation. Alternatively, aerosols may complement control achieved by insect-resistant packaging.

7. Conclusions

The use of aerosol technology for insect control in food-storage and food-processing facilities is gaining popularity as a viable alternative to expensive methods of insect disinfestation such as fumigation and heat treatment. It has several advantages over other methods of insect disinfestation. Although the aerosol technology has been used by the food-storage and food-processing industry for many decades, only recently has there been a renewed interest in generating data on aerosol distribution, efficacy, and residual activity for various existing and new aerosol formulations of insecticides in both laboratory and field settings. The reason for the renewed interest can be attributed to the phase out of methyl bromide, because of its adverse effects on stratospheric ozone, and companies embracing cost-effective and reduced-risk technologies. There is room for additional information on several areas related to insect management with aerosol applications, such as the effect of temperature on aerosol efficacy. Other areas include the effect of sanitation and pretreatment of cracks and crevices with piperonyl butoxide on improving the effectiveness of aerosol applications for insect management in food-storage and food-processing facilities. Also, there is limited information on the effects of exposure to sublethal doses of aerosols during application on stored-product insect biology and reproduction and its influence on population rebounds. Similarly, the effects of exposure to sublethal doses of aerosols during application on stored-product insects physiology (levels of detoxifying enzymes) warrants further study. The information on the waiting period required between two subsequent aerosol treatments is necessary to exploit the advantage of insects being under physiological stress from the previous aerosol exposure for effective management of insect in food-processing facilities. In conclusion, aerosols will continue to play an important role in the management of stored-product insects in food-storage and food-processing industry for the foreseeable future.

8. Acknowledgement

This article is contribution number 12-160B of the Kansas Agricultural Experiment Station.

9. References

Anonymous. 2004. Notice of proposed rulemaking-protection of stratospheric ozone: process for exempting critical uses from the phase out of methyl bromide. Federal Register 69: 52365-52402.

Arbogast, R. T., S. R. Chini, and J. E. McGovern. 2005. *Plodia interpunctella* (Lepidoptera: Pyralidae) spatial relationship between trap catch and distance from a source of emerging adults. Journal of Economic Entomology 98: 326-333.

Arbogast, R. T., P. E. Kendra, R. W. Mankin, and J. E. McGovern. 2000. Monitoring insect pest in retail stored by trapping and spatial analysis. Journal of Economic Entomology 93: 1531-1542.

Arbogast, R. T., P. E. Kendra, R. W. Mankin, and R. C. McDonld. 2002. Insect infestation of a botanicals warehouse in north-central Florida. Journal of Stored Products Research 38: 349-363.

Arthur, F.H. 1988. Evaluation of an aerosol formulation containing cyfluthrin against three species of stored-product insects. Journal of Entomological Science 23: 257-263.

Arthur, F.H. 1993. Evaluation of prallethrin aerosol to control stored product insect pests. Journal of Stored Products Research 29: 253-257.

Arthur, F.H. 2000a. Impact of accumulated food on survival of *Tribolium castaneum* on concrete treated with cyfluthrin wettable powder. Journal of Stored Products Research 36: 15-23.

Arthur, F.H. 2000b. Impact of food source on survival of red flour beetles and confused flour beetles (Coleoptera: Tenebrionidae) exposed to diatomaceous earth. Journal of Economic Entomology 93: 1347-1356.

Arthur, F.H. 2008. Aerosol distribution and efficacy in a commercial food warehouse. Insect Science 15: 133-140.

Arthur, F.H. 2010. Residual efficacy of aerosols to control *Tribolium castaneum* and *Tribolium confusum*, pp. 789-792. *In* M. O. Carvalho, C. S. Fields Alder, F. H. Arthur, C. G. Athanassiou, J. F. Campbell, F. Fleurat-Lessard, P. W. Flinn, R. J. Hodges, A. A. Isikber, S. Nvarro, R. T. Noyes, J. Riudavets, K. K. Sinha, G. R. Thorpe, B. H. Timlick, P. Trematerra, N. D. G. White [eds.], Proceedings of the 10th international working conference on stored product protection, Estoril, Portugal, Julius Kühn Archiv Nr. 425.

Arthur, F.H., and J. F. Campbell. 2008. Distribution and efficacy of pyrethrin aerosol to control *Tribolium confusum* (Coleoptera: Tenebrionidae) in food storage facilities. Journal of Stored Products Research 44: 58-64.

Arthur, F.H., and H. B. Gillenwater. 1990. Evaluation of esfenvalerate aerosol for control of stored product insect pests. Journal of Entomological Science 25: 261-267.

Arthur, F.H., J. L. Zettler, and W. R. Halliday. 1988. Insecticide resistance among populations of almond moth and Indianmeal moth (Lepidoptera: Pyralidae) in stored peanuts. Journal of Economic Entomology 81: 1283-1287.

Attfield, J. G., and D. A. Webster. 1966. Dichlorvos. Chemistry and Industry 7: 272-278.

Bernhard, K. M., and G. W. Bennett. 1981. Ultra-low-volume applications of synergized pyrethrins for stored-product insect control. Journal of Economic Entomology 74: 572-576.

Boina, D., B. Subramanyam, and S. Alavi. 2008. Dynamic model for predicting survival of mature larvae of *Tribolium confusum* during facility heat treatments. Journal of Economic Entomology 101: 989-997.

Bond, E.J., T. Dumas, and S. Hobbs. 1984. Corrosion of metals by the fumigant phosphine. Journal of Stored Products Research 20: 57-63.

Campbell, J. F., and C. Runnion. 2003. Patch exploitation by female red flour beetles, *Tribolium castaneum*. Journal of Insect Science 3: 20, available online: insectscience.org/3.20.

Campbell, J. F., F. H. Arthur, and M. A. Mullen. 2004. Insect management in food processing facilities, pp 240-295. *In* S. Taylor [ed.], Advances in food and nutrition research, vol 48, Elsevier, New York.

Campbell, J. F., M. A. Mullin, and A. K. Dowdy. 2002. Monitoring stored-product pests in food processing plant with pheromone trapping, contour mapping, and mark-capture. Journal of Economic Entomology 95: 1089-1101.

Campbell, J. F., S. Prabhakaran, B. Schneider, and R. T. Arbogast. 2003. Critical issues in the development and interpretation of pest monitoring programs for food processing facilities, pp. 121-127. *In* P. F. Credland, D. M. Armitage, C. H. Bell, P. M. Cogan, and E. Highley [eds.], Advances in Stored Product Protection, Proceedings of the 8th International Working Conference on Stored Product Protection, 22-26 July 2002, York, United Kingdom.

Childs, D.P. 1967. Cigarette beetle control in warehouses with HCN and dichlorvos. Journal of Economic Entomology 60: 263-265.

Childs, D. P, G. L. Phillips, and A. R. Press, Jr. 1966. Control of the cigarette beetle in tobacco warehouses with automatic dichlorvos aerosol treatments. Journal of Economic Entomology 59: 261-264.

Cogburn, R.R., and R. A. Simonaitis. 1975. Dichlorvos for control of stored-product insects in port warehouses: low-volume aerosols and commodity residues. Journal of Economic Entomology 68: 361-365.

Fields, P.G., and N. D. G. White. 2002. Alternatives to methyl bromide treatments for stored-product and quarantine insects. Annual Review of Entomology 47: 331-359.

Gillenwater, G. B., and P. K. Harein. 1964. A dispenser designed to provide large quantities of insecticide vapor. Journal of Economic Entomology 57: 762-764.

Gillenwater, H.B., P. K. Harein, E. W. Loy, Jr., J. F. Thompson, H. Laudani, and G. Eason. 1971. Dichlorvos applied as a vapor in a warehouse containing packaged food. Journal of Stored Products Research 7: 45-56.

Hagstrum, D.W., and P. W. Flinn. 1992. Integrated pest management of stored-grain insects, pp. 399-408. *In* D. B. Sauer [ed.], Storage of cereal grains and their products, 4th edn., AACC, St. Paul, MN, USA.

Halliday, W.R., F. H. Arthur, and J. L. Zettler. 1988. Resistance status of the red flour beetle (Coleoptera: Tenebrionidae) infesting stored peanuts in the southestern United States. Journal of Economic Entomology 81: 74-77.

Halliday, W. R., N. O. Morgan, and R. L. Kirkpatrick. 1987. Evaluation of insecticides for control of stored-product pests in transport vehicles. Journal of Entomological Science 22: 224-236.

Harein, P. K., H. B. Gillenwater, and E. G. Jay. 1970. Dichlorvos: Methods of dispensing, estimates of concentration in air, toxicity to stored-product insects. Journal of Economic Entomology 63: 1263-1268.

Harein, P. K., H. B. Gillenwater, and G. Eason. 1971. Dichlorvos space treatment for protection of packaged flour against insect infestation. Journal of Stored Products Research 7: 57-62.

Jay, E. G., H. B. Gillenwater, P. K. Harein. 1964. The toxicity of several dichlorvos (DDVP) and naled formulations to the adult confused flour beetle. Journal of Economic Entomology 57: 415-416.

Jenson, E.A., F. H. Arthur, and J. R. Nechols. 2010a. Efficacy of an esfenvalerate plus methoprene aerosol for the control of eggs and fifth instars of *Plodia interpunctella* (Lepidoptera: Pyralidae). Insect Science 17: 21-28.

Jenson, E.A., F. H. Arthur, J. R. Nechols. 2010b. Methoprene and synergized pyrethrins as aerosol treatments to control *Plodia interpunctella* (Hübner), the Indian meal moth (Lepidoptera: Pyralidae). Journal of Stored Products Research 46: 103-110.

Kirkpatrick, R.L., and H. B. Gillenwater. 1979. Toxicity of selected dusts and aerosols to three species of stored-product insects. Journal of Entomological Science 14: 334-339.

Kirkpatrick, R.L., and H. B. Gillenwater. 1981. Toxicity of selected insecticidal aerosols, dusts, and sprays to two species of stored-product insects. Journal of Entomological Science 16: 175-180.

Mahroof, R. M., B. Subramanyam, and D. Eustace. 2003. Temperature and relative humidity profiles during heat treatment of mills and its efficacy against *Tribolium castaneum* (Herbst) life stages. Journal of Stored Products Research 39: 555-569.

Mondal, K. A. M. S .H., and S. Parween. 2000. Insect growth regulators and their potential in the management of stored-product insect pests. Integrated Pest Management Reviews 5: 255-295.

Peckman, P. S., and F. H. Arthur. 2006. Insecticide space treatments in food plants, pp. 175-182. *In* J. Heaps [ed.], Insect management for food storage and processing, 2nd edn., AACC, Minneapolis, MN, USA.

Phillips, T. W., and J. E. Throne. 2010. Biorational approaches to managing stored-product insects. Annual Review of Entomology 55: 375-397.

Press, A. F., and D. P. Childs. 1966. Control of the tobacco moth with dichlorvos. Journal of Economic Entomology 59: 264-265.

Roesli, R., B. Subramanayam, F. J. Fairchild, and K. C. Behnke. 2003. Trap catches of stored-product insects before and after heat treatment in a pilot feed mill. Journal of Stored Products Research 39: 521-540.

Sutton, A. E., F. H. Arthur, K. Y. Zhu, J. F. Campbell, and L. W. Murray. 2011. Residual efficacy of synergized pyrethrin + methoprene aerosol against larvae of *Tribolium castaneum* and *Tribolium confusum* (Coleoptera: Tenebrionidae). Journal of Stored Products Research 47: 399-406.

Tenhet, J. N., C. O. Bare, D. P. Childs, and W. F. Durham. 1957. Studies of DDVP for control of cigarette beetles in tobacco warehouses. U. S. Department of Agriculture, Marketing Research Service, AMS 214, p. 15.

Tenhet, J. N., C. O. Bare, and D. P. Childs. 1958. Further studies on the control of the cigarette beetle with DDVP. Tobacco Science 147: 106-110.

Toews, M. D., F. H. Arthur, and J. F. Campbell. 2009. Monitoring *Tribolium castaneum* (Herbst) in pilot-scale warehouses treated with β-cyfluthrin: are residual insecticides and trapping compatible. Bulletin of Entomological Research 99: 121-129.

Toews, M. D., J. F. Campbell, and F. H. Arthur. 2006. Temporal dynamics and response to fogging or fumigation of stored-product Coleoptera in a grain processing facility. Journal of Stored Product Research 42: 480-498.

Toews, M. D., J. F. Campbell, F. H. Arthur, and M. West. 2005. Monitoring *Tribolium castaneum* (Coleoptera: Tenebrionidae) in pilot-scale warehouses treated with residual applications of (*S*)-hydroprene and cyfluthrin. Journal of Economic Entomology 98: 1391-1398.

Toews, M.D., J. F. Campbell, and F. H. Arthur. 2010. The presence of flour affects the efficacy of aerosolized insecticides used to treat the red flour beetle, *Tribolium castaneum*. Journal of Insect Science 10: 196 available online: insectscience.org/10.196.

Zettler, J. L. 1990. Phosphine resistance in stored product insects in the United States. Journal of Stored Products Research 26: 1075-1081.

10

The Sophisticated Peptide Chemistry of Venomous Animals as a Source of Novel Insecticides Acting on Voltage-Gated Sodium Channels

Peigneur Steve and Tytgat Jan
Laboratory of Toxicology, University of Leuven (K.U.Leuven)
Belgium

1. Introduction

Arthropods pests are considered as a global health treat since they are responsible for the transmission of several new and reemerging human diseases such as malaria, dengue and yellow fever (mosquitoes), Lyme disease, ehrlichiosis and tularemia (ticks). Furthermore, arthropods pests destroy 20-30% of the world's food supply every year. Evidently, modern agriculture still depends strongly on the use of insecticides. The majority of insecticides used today act on one of the following neuronal targets: acetylcholinesterase, the nicotinic acetylcholine receptor, the γ-aminobutyric acid (GABA)-gated chloride channel and the voltage-gated sodium channel (Na_V). This review will focus on Na_V channels as possible targets for insecticides.
For over 50 years, synthetic pyrethroids, which are analogous of the natural occurring insecticidal components of *Chrysanthemum* flowers, have been used as classical industrial pesticides. The public awareness of the health hazards and environmental damage, due to their low insect-selectivity, has complicated the use of these pesticides. More importantly, the intensive use of DDT and other pyrethroids has facilitated the development of resistance against these compounds. The so-called knockdown resistance, in which insects have reduced their sensitivity towards pyrethroids by point mutations in their target site, heralded the loss of major classes of insecticides. As a consequence, there is an urgent need for new, potent and insect-selective insecticides. A need that could be fulfilled by insecticidal neurotoxins derived from venomous animals.

2. The sodium channel structure and function

Voltage-gated sodium channels (Na_V channels) are transmembrane protein complexes constituted of an α-subunit of approximately 260 kDa which can be associated with up to four auxiliary β-subunits (β1-4) of 30 to 40 kDa. The pore-forming α-subunit alone is sufficient to obtain sodium current, however co-expression of β-subunits modifies expression level, kinetics and voltage dependence of channel gating (Yu & Catterall, 2003). The α-subunit is organized in four homologous domains (DI-IV). Each domain contains six putative transmembrane segments (S1-S6) connected by extracellular or intracellular loops

(fig. 1A). The S4 segments are the most conserved segments and they contain a basic residue, either lysine or arginine, in every third position. These positive charged S4 segments are believed to function as voltage sensors. They transport gating charges by moving outward upon membrane depolarization and as such initiating the voltage dependent activation which results in the opening of the channel. The selectivity filter and pore are formed by the transmembrane segments S5 and S6 together with the re-entrant segments that are part of the loop which connects the S5 and S6 of each domain. Folding of the domains in a clockwise orientation, in which domain I and IV are in close proximity of each other, leads to the formation of the outer vestibule and the selectivity filter (Catterall, 2000; Chanda & Bezanilla, 2002). The short intracellular linker that connects the domains III and IV contains a highly conserved sequence of three hydrophobic residues (isoleucine, phenylalanine and methionine) or IFM motif. Sodium channel inactivation is mediated by this hydrophobic motif since it serves as an inactivation gate crucial for causing fast inactivation by binding to a receptor. This inactivation gate receptor is located near or within the intracellular mouth of the sodium channel pore. It has been shown that several residues in the intracellular loop that connects IIIS4-S5 and in the loop connecting IVS4-S5 are contributing to the inactivation gate receptor (Dong, 2007; Yu & Catterall, 2003).

Nine different mammalian sodium channel isoforms have been cloned, characterized and functionally expressed. These sodium channel isoforms exhibit distinct expression patterns in skeletal and cardiac muscle tissues and in the central and peripheral nervous systems (Goldin, 1999). $Na_V1.1$, $Na_V1.2$, $Na_V1.3$ and $Na_V1.6$ are expressed in the central nervous system (CNS), whereas $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$ are predominantly expressed in the peripheral nervous system (PNS). $Na_V1.4$ is expressed in skeletal muscles, while $Na_V1.5$ is also known as the cardiac muscle isoform. The functional and pharmacological diversity of the mammalian Na_V channels is primarily resulting from the expression of multiple genes (Goldin *et al.*, 2000). The selective expression of different sodium channel genes significance the specialized function of sodium channels in various mammalian tissues and cell types (Yu & Catterall, 2003). Their specialized function results from the fact that each mammalian sodium channel α-subunit isoform features distinct electrophysiological properties such as unique gating kinetics (Dong, 2007; Goldin, 2001).

Voltage-gated insect sodium channels closely resemble their mammalian counterparts in electrophysiology, ion conductance and also in overall structure. However, they do differ in amino acid sequence and therefore in their pharmacological diversity and flexibility (Zlotkin, 1999). Furthermore, the insect-selective action of pesticides such as pyrethroids and DDT and moreover, the high specificity for either mammalian or insect Na_V channels displayed by neurotoxins from scorpion, spider and sea anemone venoms have evidenced the existence of a pharmacological distinction between mammalian and insect Na_V channels.

3. Structural comparison between mammalian and insect Na_V channels

Two genes putatively encoding for Na_V channels, *DSC1* and *para*, were isolated from *Drosophila melanogaster*. Later on, it was shown that DSC1 encodes for a Ca^{2+}-selective cation channel and not for a sodium channel. The first insect Na_V channel encoding gene, *para*, was identified from a genomic DNA library in studies using mutants with a temperature sensitive paralytic phenotype (Loughney *et al.*, 1989). The gene has been cloned and upon functional expression in *Xenopus laevis* oocytes it was demonstrated that *para* indeed encodes for a voltage-gated sodium channel ($DmNa_V1$) (Feng *et al.*, 1995; Warmke *et al.*, 1997).

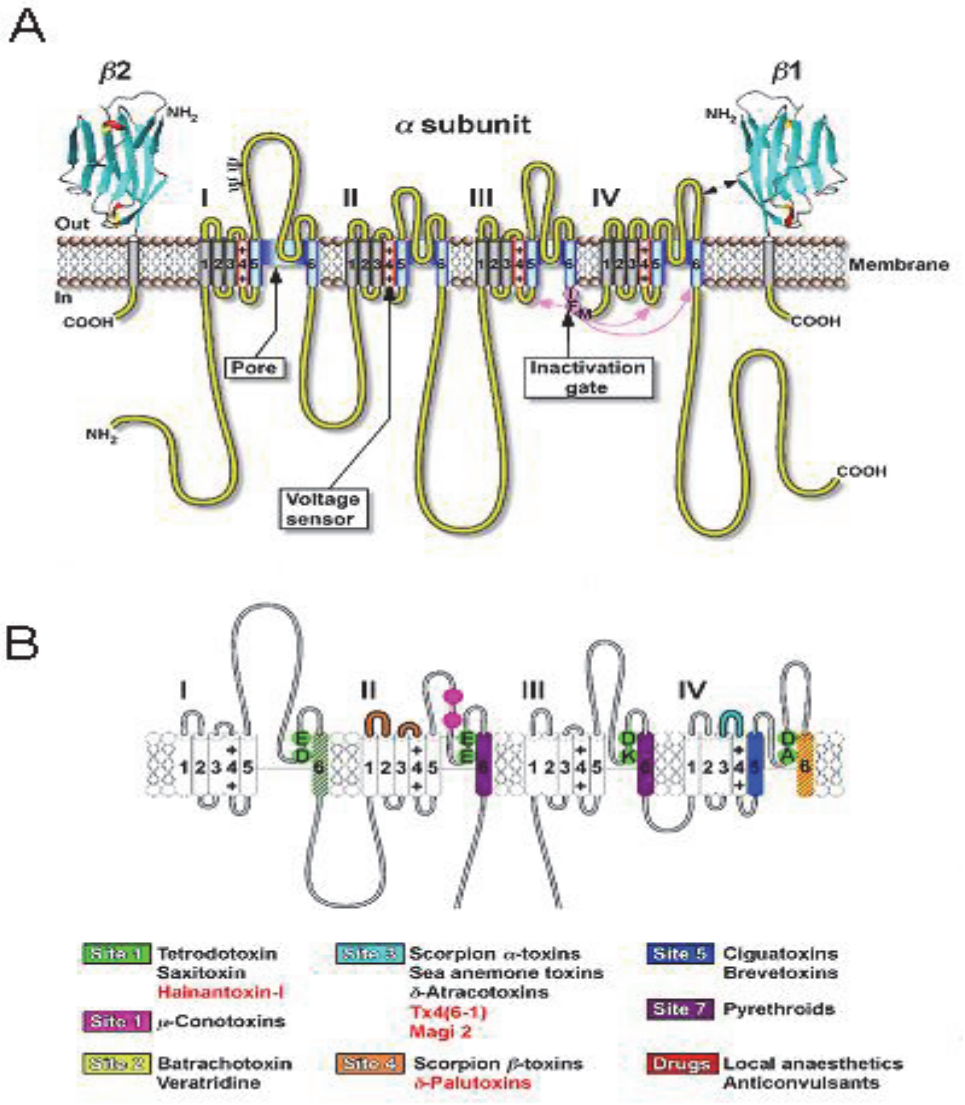


Fig. 1. Molecular structure and the neurotoxin receptor sites of Na_V channels. (A) Schematic two-dimensional representation of the functional α-subunit and the auxiliary β-subunits. (B) Identification of known neurotoxin receptor sites on Na_V channels. Green circles represent the outer (EEDD) and inner (DEKA) rings of amino acid residues that form the ion selectivity filter and the proposed neurotoxin receptor site-1. In the case of receptor sites 3 and 4, only areas where there is more than a five-fold increase in binding affinity are highlighted. Insect-selective spider toxins are highlighted in red text. Figures were adapted from (Nicholson, 2007).

Screening of the *Drosophila* genome revealed that no other genes were similar to *para*, suggesting that *para* is the only gene encoding the sodium channel in *Drosophila* and presumably also in other species (Littleton & Ganetzky, 2000). Electrophysiological recordings in different insect neurons have shown the presence of distinct sodium currents, suggesting the existence of sodium channels with differing properties (Defaix & Lapied, 2005; Grolleau & Lapied, 2000). Recent studies have shown that the occurring heterogeneity in sodium channel properties in insects is generated by extensive alternative splicing and RNA editing of the *para* gene transcript (Dong, 2007). Thus, it seems that insects rely on alternative splicing and RNA editing to produce a functional diversity of sodium channels, whereas mammals depend on multiple sodium channel genes to generate channels with unique gating properties (Dong, 2007; Yu & Catterall, 2003). An increasing number of othologous *para* genes have been identified in agricultural and medical important insect species (Soderlund & Knipple, 2003). Unfortunately, only partial cDNA clones could be obtained in most cases. Up to date, the full length cDNA clones for only 3 orthologous *para* genes have been identified: in the housefly *Musca domestica* (Vssc1), the German cockroach *Blattella germanica* ($BgNa_V$) and the mite *Varroa destructor* ($VmNa_V$) (Dong, 1997; Ingles *et al.*, 1996; Wang *et al.*, 2003). All 3 genes generate voltage-dependent sodium currents when heterologously expressed in oocytes (Du *et al.*, 2009; Ingles *et al.*, 1996; Tan *et al.*, 2002b). A functional characterization of 20 $BgNa_V$ splice variants demonstrated that, similar to *para*, RNA editing and alternative splicing results in an astonishing diversity of Na_V channels with distinct expression levels and varying kinetics of gating.

An insecticidal counterpart of the mammalian auxiliary β-subunit has also been identified in the Drosophila genome. The temperature-induced paralysis locus E (*TipE*) encodes for a small transmembrane protein consistent of two transmembrane segments which are connected by a large extracellular loop and intracellular amino and carboxyl termini (Dong, 2007; Feng *et al.*, 1995). *TipE* increases the sodium peak current, alters the kinetics of fast inactivation and changes the pharmacology of $DmNa_V1$ when it is co expressed with this para α-subunit (Warmke *et al.*, 1997). Furthermore, TipE plays an important assisting role in the trafficking of the α-subunit from the endoplasmic reticulum to the membrane and the incorporation in the membrane (Moore *et al.*, 2000).

Four *TipE* homologs have been characterized in *D. melanogaster* (*TEH1-4*) (Derst *et al.*, 2006). Similar to TipE, coexpression of TEH1-3 in oocytes results in increased sodium peak currents. Furthermore, it was shown that TEH1 shifts the voltage-dependent inactivation and it alters the rate of recovery from inactivation of $DmNa_V1$. TEH1 is only expressed in the central nervous system while TEH2-4 are widely expressed in both neuronal and non-neuronal tissues (Derst *et al.*, 2006). This might imply that these TEH auxiliary subunits are involved in specific regulation of sodium channels in wide variety of insect cells (King et al., 2008). An orthologous *TipE* gene has been cloned from the *Musca domestica* (Vssc β) (Lee *et al.*, 2000). Up to date, no ortholog has been identified in the German cockroach. However, it has been shown that co-expression of TipE with $BgNa_V$ or with Vssc1 in both cases resulted in an enhanced expression in oocytes (Lee *et al.*, 2000; Tan *et al.*, 2002a). These results suggest that the auxiliary subunits are functionally conserved among different insect species. A recent study has shown that that the channel affinity of the conotoxin MrVIB, a mammalian sodium channel blocker isolated from the cone snail *Conus marmoreus*, is strongly influenced by the co expression of β-subunits (Wilson *et al.*, 2011).

Their high conservation among different insect species, their wide expression in distinct tissues together with the observation that they are involved in the neurotoxin-channel

interaction raises the intriguing question whether the insect auxiliary subunits might represent interesting new phyla-selective targets for neurotoxin insecticides.

4. Rationale for the use of insect Na_V channels as targets for insecticides

Na_V channels mediate the increase in sodium permeability during the initial rapidly raising phase of the action potential making them a crucial component in the generation and propagation of action potentials in neurons and most electrically excitable cells. Because of this key role in the excitability of biological systems, Na_V channels are one of the foremost targets of venomous animals. These venoms are complex cocktails that have evolved to paralyze or to kill arthropod preys. Mutations and positive selection have lead to an optimization of the venom compounds which resulted in toxins able to act highly specific and very potently upon their target.

Further justification for the use of Na_V channels as targets for the development of novel insecticides is delivered by the pharmacological flexibility of Na_V channels. This arises for instance from the existence of a large number of sodium channel binding sites (fig; 1B). To date, seven neurotoxin binding sites have been identified, potentiating a broad diversity of insecticidal targets. Although the identification and characterization of the distinct receptor sites were mainly determined using vertebrate preparations, similar receptor sites have been shown for insect neuronal membranes (Gordon *et al.*, 2007). The large number of Na_V channel binding sites is much more than thus far described for other possible ion channel targets such as Ca_V and K_V channels. Several studies using insect-selective toxins from different venomous animals have underlined that even though there is an overall structural similarity with the mammalian isoforms, insect Na_V channels do exert a distinct pharmacological diversity compared to their mammalian counterparts. Furthermore, the allosteric coupling of the binding sites provides cooperative aspects which possess far-reaching practical, economical and ecological agro-technical implications (Nicholson, 2007; Zlotkin, 1999). As such, a synergistic mixture, constituted of 2 insecticides acting on distinct but allostericly coupled sites, will allow a significant decrease in the required dosages and concentrations of the 2 constituting insecticides and thus reducing production costs. Moreover, the combinatorial use of insecticides will delay the onset of resistance (Nicholson, 2007; Zlotkin, 1999).

It should be critically noted that there is no evidence to reason why Na_V channel are a more suitable target for developing new insecticides compared to other ion channels such as Ca_V or K_V. Given the fact that Na_V and Ca_V channels belong to the same superfamily of structural related voltage-gated ion channels, it is most likely that insect Ca_V channels present a similar broad panel of potential binding sites for insecticide development. Certainly since at least 3 distinct classes of insect Ca_V channels have been identified (Jeziorski *et al.*, 2000). However, due to a lack of functionally cloned insect Ca_V channels and a limited number of accessible and well-characterized insect neuron preparations, does the number of potential new insecticides acting on Ca_V channels remain scarce (King *et al.*, 2008).

5. Toxins isolated from venomous animals acting selective on insect Na_V channels

Venomous animals such as scorpions, spiders and sea anemones have become medicinal chemists of unprecedented skills by evolving their peptide chemistry and

neuropharmacology in order to develop components that insure complete shut down of the nervous system of their arthropod prey. Peptide neurotoxins that target ion channels are abundantly represented in these venoms. Specifically toxins acting on Na_V channels provide venomous animals the possibility to induce a rapid paralysis upon envenomation. These toxins, thanks to their insect selectivity, are potential new lead compounds for the development of a novel generation insecticides. All insect-selective neurotoxins characterized to date are selectively binding to three of the seven known neurotoxin sites on insect Na_V channels. Therefore these sites can be considered as potential insecticide targets (King *et al.*, 2008).

5.1 Spider venoms

Over 42,000 species of spiders have already been described worldwide (Platnick, 2000). They are divided in 110 families which are classified in the order Araneae within the Arachnida class, a group that also includes scorpions, mites and ticks. Araneae comprise three suborders: the non-venomous *Mesothelae*, the *Mygalomorphae* or tarantulas and the *Araneomorphae*.

Only a minority of spider species are capable of inflicting clinically significant or sometimes fatal envenomations. These species include the *latrodectism* or widow spiders, the *loxoscelism* or recluse spiders and certain *mygalomorph* such as the Australian funnel web and mouse spiders (Billen *et al.*, 2008; Isbister & White, 2004). Among the large number of species described, only of about 100 species has the venoms been studied. Venom composition is highly species-specific and varies depending on factors such as sex, nutrition, natural habitat and climate (Kuhn-Nentwig *et al.*, 2004; Mebs, 2002). Spiders have optimized their venoms as complex, chemical mixtures containing a variety of biological active substances serving the general purposes for both attacking (killing or paralyzing prey) and protecting (defending against competitors) (Mebs, 2002; Vassilevski *et al.*, 2009). These venoms can be divided into two groups based on the character of their function: neurotoxic and necrotic or cytolytic. This review will only focus on the neurotoxic group and more specific on the neurotoxins acting on Na_V channels. The complete molecular diversity of spider venoms has recently been very well reviewed in (Vassilevski *et al.*, 2009).

Spider neurotoxins channels are low molecular weight polypeptides. Even though peptides devoid of disulfide bridges have been reported, the majority of these neurotoxins are small disulfide rich peptides, containing 6 to 12 cysteine residues (Billen *et al.*, 2008; Pimenta & De Lima, 2005). Spider neurotoxins which target Na_V channels are in general peptides compromising 31 to 41 amino acid residues which are cross-linked by three to four disulfide bridges. Depending on their different cysteine arrangements and structural characteristics, these peptides can be divided into two distinct structural motif families: the inhibitory cysteine knot (ICK) motif and the disulfide-directed β-hairpin (DDH) scaffold (fig. 2) (Craik *et al.*, 2001; Norton & Pallaghy, 1998).

Both panels schematically represent the structural motifs of the cystine-knot folding and possible addition of the third β-sheet. β-sheets are shown as gray arrows and disulfide bridges connecting cysteine residues are shown as dark gray lines with roman numerals. The dark arrow (β1) in the right panel represents the additional β-sheet not always present in ICK spider toxins. Figure was adapted from (Nicholson, 2007).

The majority of spider toxins contain the ICK motif. It is interesting to note that peptides with the ICK motif can be found in very diverse sources such as animals, plants, fungi and even viruses. The following arrangement of disulfide bonds is observed in all peptides

belonging to this structural family: C^1-C^4, C^2-C^5, C^3-C^6. These ICK containing peptides are further characterized a β-hairpin and by the presence of a so called 'knot: the first two disulfide bridges (C^1-C^4, C^2-C^5) form a spatial ring which is penetrated by the third disulfide bridge (C^3-C^6) (Craik *et al.*, 2001; Vassilevski *et al.*, 2009). Even within a structural motif family are the biological activities diverse. Spider neurotoxins possessing the ICK motif can target proton-gated, voltage-gated or mechanosensitive channels while others exert a haemagglutination activity, protease inhibition or AMP activity (Bulet & Stocklin, 2005). The DDH scaffold is believed to be the ancestral motif from which the ICK motif has evolved. This structural motif is characterized by an arrangement of disulfide bridges as follows: C^1-C^3, C^2-C^4. Such a disulfide pattern implies that there are only two mandatory disulfide bridges that form the bulk of the hydrophobic core and that there is a formation of β-hairpins which are stabilized by these two conserved disulfide bonds (Vassilevski *et al.*, 2009).

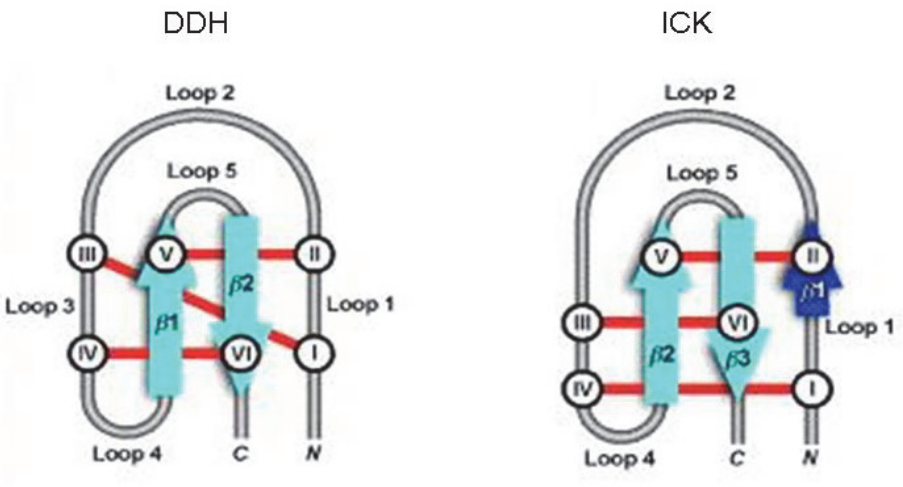


Fig. 2. Structural motifs found in insect-selective spider toxins.

Spider neurotoxins interact with Na_V channels either by binding to site 1, blocking the sodium current or by modulating the kinetics of gating by binding to site 3 and 4 (King *et al.*, 2008). Furthermore, a Na_V channel targeting family of 56-61 residue insecticidal polypeptides has been isolated from the primitive weaving spider *Diguentia canities*. Their exact interaction site with the sodium channel still remains unknown although binding studies have shown that it is unlikely that they bind to site 3 (Nicholson *et al.*, 2004). Further electrophysiological studies are awaiting in order to elucidate the binding site of these interesting insecticidal spider neurotoxins (Nicholson, 2007).

Environmental stress and evolution has led to hypermutational optimization of spider toxins resulting in mini-libraries of toxin variants allowing spiders to target slightly different versions of the same ion channel or receptor in distinct insect species (Gilles *et al.*, 2002). Therefore spider venoms contain pre-optimized insecticidal toxins, which are readily available for investigation to isolate potential lead compounds in the search for new insecticides (Nicholson, 2007).

5.2 Scorpion venoms

Scorpions belong to the most ancient group of animals on earth as they have been roaming this planet for more then 400 million years. More than 1500 different species have been described and they are classified in the order Scorpiones within the Arachnida class (Bosmans & Tytgat, 2007b). Approximately 50 species are known to cause clinical significant injuries out of which 25 are potentially fatal to humans. A scorpion sting might be trivial causing local pain only, but may also produce a very complex symptomatology of envenoming such as neurological, respiratory and cardiovascular collapse. Almost all of the lethal scorpions, except the *Hemiscorpius* species, belong to the family of the *Buthidae*. Dangerously venomous scorpions tend to have lean, delicate pincers, thin bodies and thick tails, as opposed to the large, bulky pincers, thick bodies and thin tails possessed by the non-lethal scorpions (Mebs, 2002). The lethal members of the *Buthidae* family include the genera of *Androctonus* (Northern Africa to Southeast Asia), *Centruroides* (Southwest of Northern and Central America), *Leiurus* (Middle East and Northern Africa), *Mesobuthus* (Asia), *Parabuthus* (Southern and Western Africa), *Buthus* (Mediterranean) and *Tityus* (Caribbean, Central and Southern America).

Scorpion venoms are multi component mixtures containing an unprecedented molecular diversity of pharmacologically active components such as enzymes, nucleotides, lipids, mucoproteins, biogenic amines, polypeptides and other unknown substances. The most characterized components of scorpion venoms are neurotoxins which have evolved towards a specific and specialized bioactivity aimed at efficient and successful capture of prey or defense against predators. Scorpion neurotoxins specifically target voltage-gated ion channels such as Na_V, K_V or Ca_V and other cellular receptors such as the ryanodine receptor (Zamudio *et al*., 1997). Similar to spiders, is the composition of scorpion venom highly differing between species and are factors such as sex, habitat and local conditions determining for venom specificity (Bosmans & Tytgat, 2007b; Possani *et al*., 1999). For example, scorpion neurotoxins targeting Na_V channels are much more represented in the venom from scorpions belonging to the dangerous *Buthidae* as compared to the venom of harmless species such as *Pandinus*. Moreover, the overall toxicity of scorpion venom to humans has mainly been attributed to the activity of neurotoxins affecting Na_V channels (Martin-Eauclaire *et al*., 2005).

In general, scorpion neurotoxins acting on Na_V channels are single chain polypeptides composed of 58-76 amino acids and cross-linked by four disulfide bridges. They posses a highly conserved core formed by an α-helix and two to three strands of β-sheet structural motifs, stabilized by the three intermolecular disulfide bridges (Bosmans & Tytgat, 2007b). The scorpion structural motif families compromise the βαββ family and the βααββα family which are al stabilized by the four disulfide bridges (Mouhat *et al*., 2004). Both structural motif families belong to the structural cysteine-stabilized α-helix and β-sheet (CSαβ) superfamily. Peptides belonging to the CS superfamily exhibit relatively diverse biochemical and biological functions. However, in most cases are these peptides sharing a common function as defenders of their host, as seen in animals (e.g. scorpion toxins), plants (e.g. defensins) and microorganisms (e.g. antifungal defensins). The extensive distribution of this common motif throughout diverse organisms highlights that this relatively stable and versatile scaffold has the potential to tolerate insertions, deletions and substitutions within the structure (Zhu *et al*., 2005).

According to their pharmacological profile, scorpion neurotoxins acting on Na_V channels can be divided into two major groups: α-toxins and β-toxins. Upon binding at neurotoxin

receptor site 3, α-toxins modulate the Na_V channel by slowing down the fast inactivation. β-toxins modulate the activation process by binding to neurotoxin site 4. According to their preference for mammalian or insect Na_V channels, are α-toxins further subdivided into three groups: (i) the classical α-toxins with a preference for mammalian Na_V channel isoforms; (ii) the α-like toxins which are active on both the insect and mammalian Na_V channels; (iii) the insect α-toxins which are capable of discriminating with high affinity between insect and mammalian Na_V channels and thus serve as potential candidates for the development of insecticides (Billen *et al*., 2008; Gordon *et al*., 2007; Gurevitz *et al*., 1998).

Similar to the α-toxins are the β-toxins also classified into 3 groups according to their pharmacological properties, exemplified by their preference for mammalian or insect sodium channels: (i) Mammalian-selective β-toxins are highly toxic to mammals (Martin *et al*., 1987); (ii) β-like toxins are capable of competing for binding sites on both insect and mammalian Na_V channels; (iii) Insect-selective β-toxins fail to exert any affinity whatsoever for mammalian sodium channels, even in very high concentrations (de Dianous *et al*., 1987). Exactly this complete lack of mammal activity combined with their strong insect specificity and potency makes these insect-selective β-toxins interesting lead compounds in the design of new insecticides (Gurevitz *et al*., 2007). The insect-selective β-toxins can be further subdivided into excitatory and depressant toxins according to the symptoms they evoke *in vivo*. Injection of excitatory toxins induces a fast repetitive activity of motor nerves which results in a reversible contraction paralysis (Billen *et al*., 2008; Zlotkin *et al*., 1985). These excitatory toxins differ from the other β-toxins because there is one disulfide bridge differently located and furthermore they do display extra secondary structural elements (Gurevitz *et al*., 1998; Rodriguez de la Vega & Possani, 2005). The depressant toxins cause a transient contraction followed by a slow depressant and flaccid paralysis (Karbat *et al*., 2007; Zlotkin *et al*., 1991). Remarkably, insect-selective depressant β-toxins are given the opportunity to affect mammalian channels when these channels are excited by a long preconditioning depolarizing prepulse. This potential prerequisite to affect mammalian Na_V channels possibly contributes to the observed lack of toxicity of depressant toxins towards mammals.

Predictions suggest that up to 100 000 distinct polypeptides are present in all known scorpion species. At this moment, only 1% thereof has been biochemical and functional characterized (Possani *et al*., 1999). The high number of yet unknown peptides together with the strong phyla specificity exerted by their neurotoxins validates scorpion venoms as a promising source for novel Na_V channel targeting insecticides.

5.3 Sea anemone venoms

Sea anemones are ocean dwelling, solitary animals belong to the phylum Cnidaria. There are over 1400 species described, enclosing more then 45 families which are grouped into the order Actiniaria within the Anthazoa class. Sea anemones are widely distributed around the world as they can be found from the poles to the equator. Most sea anemones live in tidal zones and in shallow water. Sea anemones generally do represent a serious risk for humans as stings usually only cause mild reactions such as inflammation, pain and edema. However, certain tropical species can deliver a more painful sting in which case often also necrosis is observed (Oliveira *et al*., 2009). Sea anemone venoms are a known pharmacological treasure of biological active compounds. The venom can be divided into two proteic groups of compounds: (i) pore-forming toxins such as hemolysins and actinoporins; (ii) neurotoxins (Beress & Beress, 1975). These neurotoxins are acting upon a

diverse panel of ion channels such as TRPV1, Na_V, K_V and acid-sensing channels (Diochot *et al.*, 2003, 2004). Of these different toxins, those that target Na_V channels are the best studied group with more then 100 known toxins (Bosmans & Tytgat, 2007a). In contrast, no more then 12 K_V channel toxins have been characterized to date. Since the beginning of last century sea anemones have been studied with an increasing interest. Although a number of sea anemone toxins have been isolated and characterized, these animals remain poorly studied in comparison with other venomous animals such as scorpions, spiders, cone snails or snakes. Based on structural differences and activity profile, the sea anemone toxins targeting Na_V channels have been subdivided into 3 groups or types (Honma & Shiomi, 2006). Type 1 and 2 toxins are polypeptides composed of 46-49 amino acid residues, stabilized by the connection 6 cysteine residues forming 3 disulfide bridges. Both type 1 and 2 toxins are believed to have evolved from the same ancestral gene (Ishida *et al.*, 1997). Consequently, type 1 and 2 toxins share the conservation of the six half-cysteines as well as several other residues thought to play a role in biological activity. Nevertheless, from a immunological point of view are these toxins distinguishable since no cross-reactivity occurs (Norton, 1991). Type 1 toxins are characterized by a core of four strands of anti-parallel β-sheets connected by two or three loops (Salceda *et al.*, 2007). Type 3 sea anemone toxins comprise 4 peptides. These toxins contain 30-32 amino acid residues, stabilized by 4 disulfide bridges (Honma & Shiomi, 2006). All 3 types of sea anemone Na_V channel toxins interact with site 3, herby altering the inactivation kinetics.

The validation of sea anemone as a potential source for new neurotoxin derived insecticides based on the existence of crustacean selective peptides in these sea anemones might seem peculiar. However, from an evolutionary point of view, it is understandable why we can encounter potential insecticidal toxins in the venom of sea anemones even when insects and sea anemones will never encounter one another (Bosmans & Tytgat, 2007a). It has been proposed that the 'Pancrustacean' taxon comprises all crustacean and hexapods (Bosmans & Tytgat, 2007a). It should herby be noted that the class Insecta is comprised in the phyla Hexapoda which is a subphylum of the phylum Arthropoda. A monophyletic 'Pancrustacea' taxon has been supported by many molecular studies (Nardi *et al.*, 2003; Shultz & Regier, 2000). In these studies is most of the subphylum Crustacea paraphyletic with respect to insects. Therefore it can be concluded that insects are descendents from crustacean ancestors and thus by definition can crustacean-selective toxins be considered as insect-selective toxins. Based on these peculiar evolutionary and pharmacological arguments have sea anemone venoms earned there status of promising source for new pesticides.

6. Neurotoxin receptor sites of Na_V channels as molecular targets of insect-selective toxins

6.1 Insect-selective inhibiters of the sodium conductance: site 1 *toxins?*

The amino acid residues that form the neurotoxin receptor site 1 are primarily located in the pore loop which is formed by the membrane dipping part of the connecting loop between S5 and S6 of each domain (Catterall *et al.*, 2007). This site is occupied by the water soluble heterocyclic guanidine tetrodotoxin (TTX) which is isolated from the tissue of at least 40 different species of puffer fish but it can also be found in mollusks, crabs, octopus and frogs (Catterall *et al.*, 2007; Hwang *et al.*, 1991). TTX exerts its strong toxicity and high fatality, also towards humans, by binding within the inner pore of the channel, physically occluding the ion pathway. Based on their sensitivity to TTX have the mammalian isoforms been divided

into TTX-sensitive ($Na_V1.1$-$Na_V1.4$, $Na_V1.6$ and $Na_V1.7$) or TTX-insensitive ($Na_V1.5$, $Na_V1.8$, $Na_V1.9$) (Narahashi, 2008).

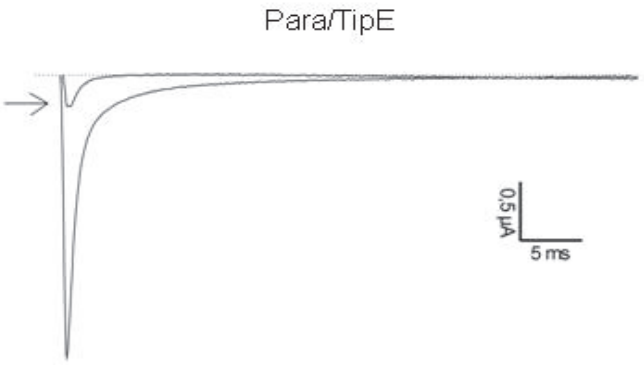


Fig. 3. Effects of site 1 toxins on insect Na_V channels. Panel represents the effects of site 1 toxins on insect Na_V channels expressed in *Xenopus laevis* oocytes. The arrow indicates the steady-state condition after application of site 1 toxin. Binding upon site 1 causes an inhibition of the sodium conductance (indicated by the arrow). Currents were evoked by 100 ms depolarizations to 0 mV, from a holding potential of -90 mV.

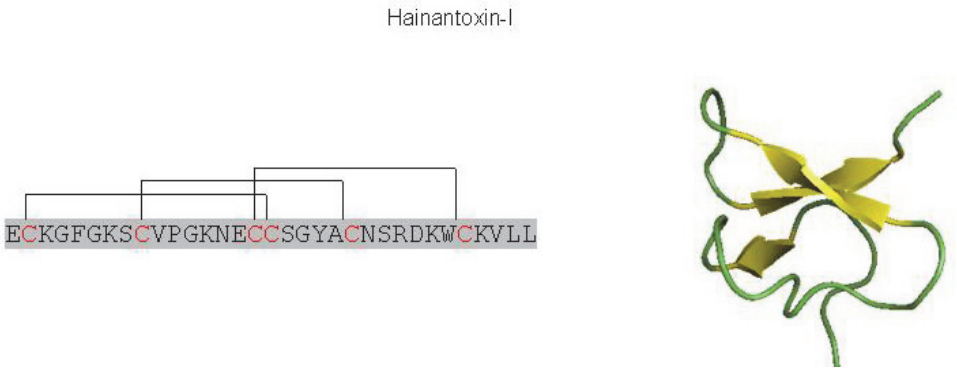


Fig. 4. Hainantoxin-I. Sequence and structural model of the insect-selective spider toxin Hainantoxin-I which is folded accordingly the ICK motif (PDB identification number: 1NIX).

Besides TTX, it has also been shown that µ-conotoxins, neurotoxin peptides isolated from Cone snail species, bind to a micro site within the neurotoxin receptor site 1 (Stephan *et al.*, 1994). Even though these µ-conotoxins are known to be highly selective and potent towards mammalian sodium channel isoforms, little investigation has been done regarding their activity towards insect sodium channels. No insect selective µ-conotoxin has been described yet. However, a recent study has reported that the δ-conotoxin TxVIA, which binds to receptor site 6 on Na_V channels, shows insecticidal activity when injected into lepidopteran (cabbage moth) and dipteran (house fly) larvae, suggesting that Cone snail neurotoxins

might have an interesting insecticidal potential (Bruce *et al.*, 2011). For example, the terrestrial molluscan crop pest, *Deroceras reticulatum* or grey field slug, is the most damaging molluscan crop pest in the UK. Annual applications of pellets containing over 250 tonnes of active ingredients are estimated to cost approx. 34 million euro per annum (Bruce *et al.*, 2011). As such it can be reasoned that Cone snail species, which have other mollusks as natural competitors, could be a yet unexplored source of neurotoxin-derived novel insecticides.

6.1.1 Site 1 toxins from spider venom

Huwentoxins and hainantoxins, isolated from the Chinese bird spiders *Ornithoctonus huwena* and *Ornithoctonus hainana* respectively, all belong to the same family of spider toxins. They are constituted of 33-35 amino acids, cross-linked by 3 disulfide bridges and folded according to the ICK motif. Both huwentoxins and hainantoxins are found to target Na_V channels (King *et al.*, 2008). Hainantoxin-I (HNTX-I) causes an inhibition of the sodium conductance without alteration of channel inactivation kinetics or of the voltage dependence of steady-state activation (Billen *et al.*, 2008). HNTX-I stabilizes the channels in the inactivated state as demonstrated by the observed hyperpolarizing shift in the voltage dependence of steady-state inactivation. Electrophysiological studies have shown that HNTX-1 displays a 15 fold-selectivity for insect Na_V channels over the mammalian channel $rNa_V1.2$. HNTX-I does not show any affinity for other mammalian Na_V channel isoforms and is therefore an interesting lead in the development of insecticides (Li *et al.*, 2003).
Huwentoxin IV and hainantoxin III-V are also capable of reducing the sodium conductance but unlike HNTX-I show these toxins affinity for both mammals and insects (Xiao & Liang, 2003). These spider toxins possibly exert there activity through an interaction with site 1 although this remains to be determined.
It has been claimed that this group of toxins is the first family of spider polypeptides capable of selectively blocking Na_V channels by occupying site 1. However, studies have evidenced that huwentoxin IV binds at site 4, trapping the voltage sensor of domain II in its inward position rather than interacting with site 1 or a distinct binding site within the extracellular pore region. Characterization of the huwentoxin IV interaction revealed that this toxin fails to induce any modification on the activation and steady-state inactivation, making it electrophysiological distinguishable from the previously described HNTX-I induced effects (Xiao *et al.*, 2008). Therefore competitive radioligand binding studies are required to confirm for each of these toxins the exact interaction site with the voltage-gated sodium channel. Notwithstanding this lack of structural data, it can be concluded that these spider toxins represent the first family to selectively block the sodium conductance and, moreover, HNTX-1 can be seen as the first insect-selective Na_V channel inhibiting peptide isolated from spider venom (Nicholson, 2007).
To date, no insect-selective neurotoxins capable of inhibiting the sodium conductance have been isolated from the venoms of scorpions or sea anemones.

6.2 Insect-selective gating modifiers of inactivation: site-3 toxins

The neurotoxin receptor 3 is mainly localized at the extracellular loop connecting the segments S3 and S4 from domain IV. It is believed that other parts of the channel such as the extracellular loops between the S5 and S6 of domain I and IV also contribute significantly to channel recognition and binding of site 3 toxins (Bosmans & Tytgat, 2007a). The voltage sensors of each domain will normally move outward under influence of the electric field

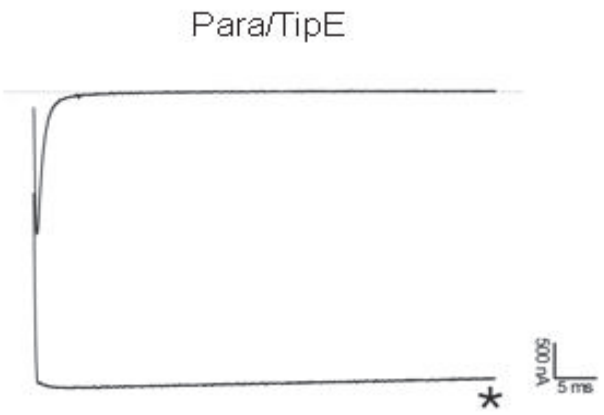


Fig. 5. Effects of site 3 toxins on insect Na_V channels. Panel represents the effects of site 3 toxins on insect Na_V channels expressed in *Xenopus laevis* oocytes. The asteriks indicates the steady-state condition after application of site 3 toxin. Binding upon site 3 causes such an extreme slowing down of inactivation that channels simply do not inactivate anymore. The result is a massive influx of Na^+ ions. Currents were evoked by 100 ms depolarizations to 0 mV, from a holding potential of -90 mV.

when the membrane is depolarized (Hille, 2001). Upon binding at site 3 these toxins trap the voltage sensing segment S4 of domain IV in its inward position. As such they prevent the normal outward movement of these voltage sensors and herewith the conformational changes necessary for fast inactivation (Catterall *et al.*, 2007).
Summarized, toxin binding at neurotoxin receptor 3 affects the coupling of activation and inactivation, resulting in a slowing down or inhibition of the fast inactivation (fig. 5). Several studies have demonstrated the functionally relevant structural differences in insect and mammalian receptor site 3 regions (Gordon *et al.*, 1996). Although more structure-function work is necessary to completely unravel these structural differences, these phyla depending differences do support the arguments to target site 3 in the search for new Na_V channel acting insecticides (Cohen *et al.*, 2006).

```
Magi 2        CMGYDIECNENLPCCKHRKLECVETSGYWWYKRKYCRPIK

Tx4(6-1)      CGDINAACKEDCDCCGYTTACDCYWSKSCKCREAAIVIYTAPKKKLTC
PnTx4(5-5)    CADINGACKSDCDCCGDSVTCDCYWSDSCKCRESNFKIGMAIRKKF-C
PnTx4-3       CGDINAACKEDCDCCGYTTACDCYWSSCKCREAAIVIYTAPKKKLTC
```

Fig. 6. Insect-selective site 3 toxins isolated from spider venom.

Comparison of the amino acid sequences of insect-selective spider toxins which target site 3. Identical residues are boxed in grey, cysteines are shown in red.

6.2.1 Site 3 toxins from spider venom

Several insect-selective spider neurotoxins are known to bind at site 3. Six spider neurotoxins (Magi 1-6) have been isolated from the Japanese funnel-web spider *Macrothele gigas* (Corzo *et al.*, 2003). Magi 1-4 compete for site 3 on insect Na_V channels with the well characterized scorpion toxin LqhαIT in radioligand binding experiments. Magi 2, which is composed of 40 amino acid residues and posses the ICK fold, shares little sequence homology with any other spider toxin. It is an insect-selective toxin as it induces paralysis in insects while it is devoted of any activity on mammals. Like other spider toxins acting on site 3, Magi 2 still awaits delineation of its structure-function relationship in order to elucidate the key residues involved in the insect-selectivity and potent channel modulation of this toxin (Nicholson, 2007). The venom of the South American 'armed' spider *Phoneutra nigriventer* has been extensively studied as this spider accounts for the majority of spider envenomations in Brazil (Borges *et al.*, 2009). Several insecticidal peptides have been isolated from the venom of *P. nigriventer*. One of them, a 48 residue long polypeptide with 5 disulfide bonds called PnTx4(6-1), was shown to exhibit its insecticidal activity through potent modulation of Na_V channels. Moreover, this toxin binds with high affinity to site 3 of insect channels but fails to bind at the mammalian Na_V channel isoforms $rNa_V1.2$ and $rNa_V1.4$, even in high concentrations (de Lima et al., 2002). Furthermore, two other toxins from *P. nigriventer*, PnTx4(5-5) and PnTx4-3, have shown to be highly insecticidal towards houseflies and cockroaches. At the same time, these toxins display no toxicity towards mammals. It has been shown that PnTx4(5-5) acts on NMDA-subtype of glutamate receptors and that PnTx4-3 inhibits glutamate intake possibly through interaction with Na_V channels (Borges *et al.*, 2009). Indeed, because of the high homology in sequence between these two toxins and PnTx4(6-1) it can be hypothesized that both toxin exert there insect-selective activity at least in part through an interaction with Na_V channels.

Other spider neurotoxins which bind to site 3 are the δ-atracotoxins, isolated from the venom of Australian funnel-web spiders. Although δ-atracotoxins modulate insect Na_V channels with high potency, they are of less interest for the development of new insecticides due to there almost equal affinity for both mammalian and insect Na_V channels (Nicholson *et al.*, 2004). Therefore this group of spider toxins will not be discussed in this review.

Magi 2 and PnTx4(6-1) are two examples of a growing group of potent insect-selective Na_V channel toxins from spiders, validating both spider venoms as source of insecticidal peptides and site 3 as a potential target of novel peptide derived insecticides.

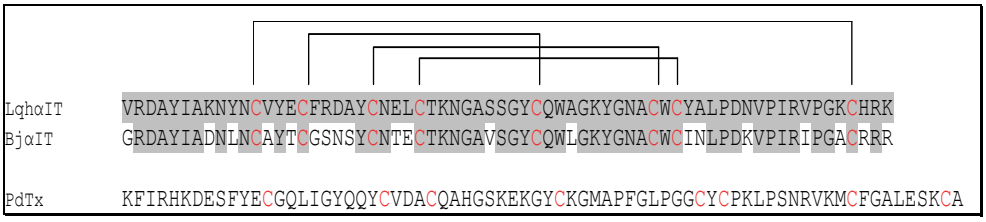


Fig. 7. Insect-selective site 3 toxins isolated from spider venom.

Comparison of the amino acid sequences of insect-selective scorpion toxins which target site 3. Identical residues are boxed in grey, cysteines are shown in red, disulfide-bonding pattern is indicated above sequences.

6.2.2 Site 3 toxins from scorpion venom

The insect α-toxins isolated from scorpion venoms are potently active on insects and at the same time importantly weak or not active on mammals. Mutagenesis studies and toxin crystal structure determinations have shed light upon the structural basis which provides these toxins the ability to discriminate between insect and mammal Na_V channels (Guan *et al.*, 2004). The insect α-toxin LqhαIT, isolated from the scorpion *Leiurus quinquestriatus hebraeus,* is the most potent and best characterized insecticidal scorpion toxin up to date (Gordon *et al.*, 2007). This peptide is 64 amino acids long and contains 8 cysteins. Similar to all scorpion neurotoxins acting on Na_V channels, LqhαIT belongs to the CSαβ structural superfamily (Tugarinov *et al.*, 1997). It serves as a marker toxin for site 3 and is widely used as radiolabeled ligand in binding studies.

More recently, BjαIT was isolated from the black scorpion *Hotentota judaica.* This toxin is an insect-selective toxin acting on site 3 of insect Na_V channels in nM range. Even a tenfold higher concentration displays only weak activity on the mammalian sodium channel isoform $Na_V1.2$ (Arnon *et al.*, 2005).

Phaiodotoxin is a new 72 amino acid long peptide with a disulfide bond pattern which is unique because of the position of the 2 cysteines forming the fourth bridge. This toxin, isolated from the Mexican scorpion *Anuroctonus phaiodactylus*, shares only 49% homology with any known scorpion toxin (Valdez-Cruz *et al.*, 2004). Interestingly, phaiodotoxin acts as an insect-selective toxin with a unique alteration of Na_V channel gating. It causes a negative shift in the voltage dependence of activation and, at the same time, a positive shift in the voltage dependence of inactivation. Both alterations of gating together results in an increased 'window current' by 225%, which is thought to be the cause of its high toxicity toward insects.

Fig. 8. Insect-selective site 3 toxins isolated from scorpion venom.

Comparison of the amino acid sequences of insect-selective sea anemone toxins which target site 3. Identical residues are boxed in grey, cysteines are shown in red, disulfide-bonding pattern is indicated above sequences.

6.2.3 Site 3 toxins from sea anemone venom

In comparison with scorpion and spider toxins, are sea anemone toxins poorly studied. However, their remarkable insect over mammalian specificity has been noticed already a long time ago (Schweitz, 1984). It was evidenced that the toxin ATX-I, isolated from the wax sea anemone *Anemonia sulcata*, could display preferential toxicity for crabs over mice (Norton, 1991). Since it has been described on a genetic level that there is a link between

crustaceans and insects, one could hypothesize that ATX-I consequently has potential as insect-selective toxin (Boore *et al.*, 1998). Another toxin from the same sea anemone, ATX-II is toxic to both insect and mammalian Na_V channels. However, its binding affinity for cockroach neuronal membranes is very high whereas its binding affinity for rat brain synaptosomes is low (Gordon *et al.*, 1996). Furthermore, ATX-II binds with extreme high potency to site 3 of insect Na_V channels, resulting in a strong slowing down of the fast inactivation (Warmke *et al.*, 1997). Sh-I from *Stichodactyla gigantea* and CgII from *Condylactis gigantea* were found to be moderately toxic to insects but were essentially non-toxic to mammals (Salgado & Kem, 1992).

Two toxins BgII and BgIII, both from *Bunodosoma granulifera*, have been extensively studied. These sea anemone toxins were studied for their activity on cloned mammalian and insect Na_V channels, dorsal root ganglia and rat brain synaptosomes (Bosmans *et al.*, 2002; Goudet *et al.*, 2001). Furthermore were these toxins also thoroughly tested in mice (Loret *et al.*, 1994). These studies revealed that both BgII and BgIII have a preference for insect channel. However, BgII has a 100-fold higher potency on insect channels compared to mammalian channels, BgIII only exert a 5-fold difference in potency of insect over mammal. This dissimilarity in insect-selectivity between both peptides is remarkable in this way that BgII and III only differ from each other in one amino acid, namely an aspargine to aspartate at position 16 (Bosmans *et al.*, 2002).

Another justification for the interest in sea anemone toxins as potential insecticides can be found in the fact that these toxins have a devastating effect on the inactivation of insect channels. Upon binding at site 3, sea anemone toxins cause such an extreme slowing down of inactivation that channels simply do not inactivate anymore (fig. 5) (Bosmans & Tytgat, 2007a).

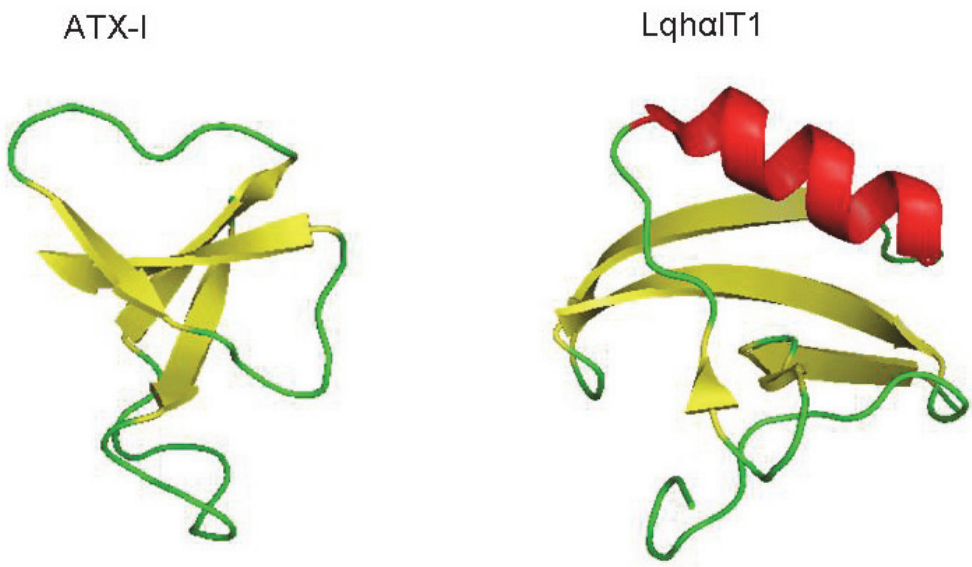


Fig. 9. Structural model of insect-selective site 3 toxins.

Insect-selective sea anemone toxins and insect-selective scorpion toxins, represented by ATX-I and LqhαIT1, respectively. Both toxins exhibit their insecticidal activity by binding upon site 3. PDB ID number:1ATX and 2YEO.

6.3 Insect-selective gating modifiers of activation: site-4 toxins

Toxin binding at site 4 causes a shift in the voltage dependence of activation towards more hyperpolarized membrane potentials and reduces the peak sodium current amplitude (Cestele *et al.*, 2006; Vijverberg *et al.*, 1984). The shift in voltage dependence of Na_V channel activating causes channels to open at, or close to, the resting potential. This increase in open channel probability leads to repetitive firing and consequently increases the influx of Ca^{2+} into the nerve terminals resulting in an increased frequency of miniature excitatory junctional potentials (King *et al.*, 2008). The alterations in channel gating are believed to be a direct result of toxin binding at site 4 leading to a trapping of the voltage sensor in its outward, activated position (Cestele *et al.*, 2001, 2006). The receptor site 4 has been primarily defined to specific residues in the extracellular loops connecting the S1-S2 and S3-S4 segments of domain II (Catterall *et al.*, 2007). However, using the scorpion β-toxin Tz1 (*Tityus zulianus*) it was shown that three residues in the pore loop of domain III are determining for the specificity of β-toxin for different sodium channel isoforms (Leipold *et al.*, 2006). A recent report showed that specific mutations in the voltage sensor of domain III enhance the binding of site 4 toxins to S4 of domain II providing evidence for the involvement of the domain III voltage sensor in the action mechanism of toxins acting on site 4 (Song *et al.*, 2011).

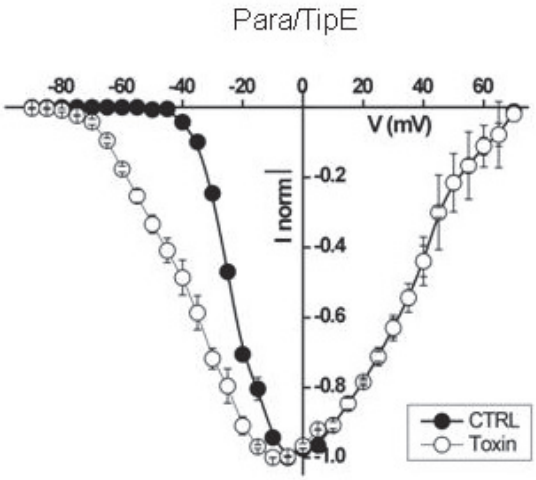


Fig. 10. Effects of site 4 toxins on insect Na_V channels. Panel represents the effects of site 4 toxins on insect Na_V channels expressed in *Xenopus laevis* oocytes. Toxin binding upon site 4 results in a shift in the voltage dependence of activation towards more negative membrane potentials, causing channels to open at potentials they normally remain closed. Currents were, from a holding potential of -90 mV, evoked by 100 ms depolarizations ranging from -90mv to 65 mV in 5 mV increments.

Fig. 11. Insect-selective site 4 toxins isolated from spider venom.

Comparison of the amino acid sequences of insect-selective spider toxins which target site 4. Identical residues are boxed in grey, cysteines are shown in red, disulfide-bonding pattern is indicated.

6.3.1 Site 4 toxins from spider venom

Insect-selective gating modifiers of activation have been isolated from spider venom. A highly selective site 4 toxin, Bs1 has been isolated from the venom of the Mexican theraposid spider *Brachypelma smithi*. Although with low potency, Bs1 could significantly shift the voltage-dependence of activation of insect channels whereas it did not affect mammalian Na_V channel isoforms (Corzo *et al.*, 2008). Another group of insect-selective site 4 spider toxins is constituted by the δ-palutoxins (*Paracoelotes luctuosus*), curtatoxins (*Hololena curta*), μ-agatoxins (*Agelenopsis aperta*) and the recently characterized β/δ-agatoxins (*Agelena orientalis*) (Billen *et al.*, 2010; Corzo *et al.*, 2000; Stapleton *et al.*, 1990). All peptides belonging to this group are structurally related as they are composed of 36-37 residues and cross-linked by 4 disulfide bridges forming an ICK motif (Nicholson, 2007). Little is known about the mechanism of action of the curtatoxin but the highly homologous μ- and β/δ-agatoxins and δ-palutoxins have been well studied. It was reported that the μ-agatoxins could shift the voltage-activation curve towards more hyperpolarized potentials. However, these toxins also slowed down the inactivation process of the sodium channels, resulting in a non-inactivating persistent current (Adams, 2004). The same observations were made for the β/δ-agatoxins and a thoroughly electrophysiological characterization of the action of these agatoxins was performed. It was concluded that agatoxins induce a bell-shaped voltage-dependent modulation of both the activation and the inactivation of insect Na_V channels, suggesting no strict correlation between the toxin binding site and its effect on channel gating (Billen *et al.*, 2010). The insect-selectivity of the δ-palutoxins was designated based on studies showing that these toxins potently modulate insect Na_V channels but fail to exert any affinity for the mammalian Na_V channel isoform $Na_V1.2$ (Ferrat *et al.*, 2005). Mutagenesis studies have confirmed that δ-palutoxins contain the same pharmacological determining key residues as the well studied site 4 toxin Bj-xtrIT. Furthermore, the δ-palutoxins compete with this depressant β-toxin Bj-xtrIT for site 4 but they fail to displace the binding of α-toxin LqhαIT from site 3 (Corzo *et al.*, 2005). Notwithstanding herewith, these toxins act as insect-selective modulators of sodium channels by slowing down the inactivation, a modulation typically seen upon toxin binding at site 3 (Corzo *et al.*, 2000). This remarkable difference in mode of action provides novel perspectives about the structural relatedness of receptor site

3 and 4. Therefore, the topological distinction between these two sites should be questioned. The structural belief that site 3 and 4 rather belong to one extended macrosite merit plausibility as the δ-palutoxins revealed that modulation of inactivation can be achieved by binding to a site which was until now, believed to be associated with alteration on channel activation (Nicholson, 2007).

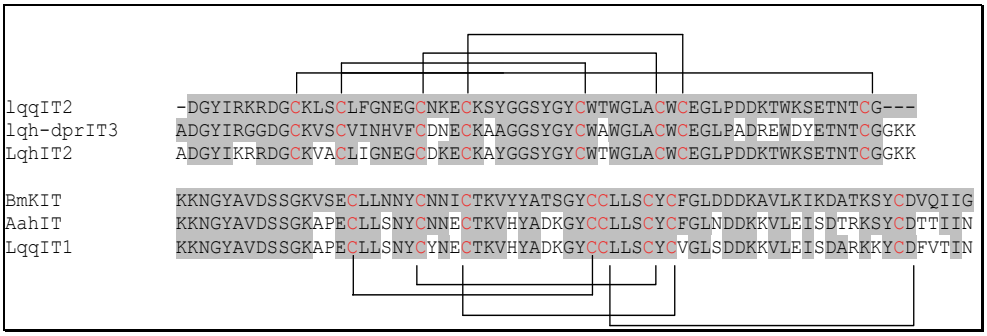


Fig. 12. Insect-selective site 4 toxins isolated from scorpion venom.

Comparison of the amino acid sequences of insect-selective scorpion toxins which target site 4. Identical residues are boxed in grey, cysteines are shown in red. The disulfide-bonding pattern is indicated above the sequences of the depressant toxins and under the sequences of the excitatory toxins, respectively.

6.3.2 Site 4 toxins from scorpion venom

Similar to spiders are scorpions also capable of producing toxins which recognize the neurotoxin receptor site 4. Toxins belonging to the class of insect-selective β-toxins are long chain peptides composed of 58 to 76 amino acids, cross-linked by four disulfide bridges. They belong to the structural superfamily of cysteine stabilized α/β motif containing proteins. The insect-selective β-toxins can be further subdivided into excitatory and depressant toxins according to the symptoms they evoke *in vivo*. AahIT from *Androctonus australis Hector*, LqqIT1 from *Leiurus quinquestriatus quinquestriatus,* Bj-xtrIT isolated from *Hotentota judaica* and BmKIT from *Buthus martensii* Karsch belong to excitatory insect-selective β-toxins (Billen *et al.*, 2008; Froy *et al.*, 1999; Zlotkin *et al.*, 1985). These excitatory toxins differ from the other β-toxins because there is one disulfide bridge differently located and furthermore they do display extra secondary structural elements (de la Vega & Possani, 2007). AahIT induces repetitive firing of action potentials as a result of activation of Na_V channels at lower membrane potentials, explaining the typical contractile paralysis observed in insects injected by excitatory insect-selective β-toxins such as AahIT.

The depressant toxins cause a transient contraction followed by a slow depressant and flaccid paralysis (Karbat *et al.*, 2007; Zlotkin *et al.*, 1991). Current clamp experiments have shown that peptides belonging to this group suppress the evoked action potentials as a result of strong depolarization of the membrane, causing an inability of axons to generate action potentials (Strugatsky *et al.*, 2005). Representatives of this group are LqqIT2 from *Leiurus quinquestriatus quinquestriatus*, BjIT2 from *Buthotus judaicus* and the highly potent and insecticidal toxins lqh-dprIT3 and LqhIT2, isolated from the scorpion *Leiurus quinquestriatus hebraeus* (Zlotkin *et al.*, 1993). It is interesting to note that LqqIT2 did not only cause a

hyperpolarizing shift in the activation of channels, it also affected the inactivation and the ion selectivity (Bosmans *et al.*, 2005). Therefore, the high insecticidal action of this toxin may be attributed to a combined modification of gating kinetics, resulting in Na_V channels that open at more negative membrane potentials and inactivate not normally and thus, display a significantly increased open probability. Remarkably, insect-selective depressant β-toxins are given the opportunity to affect mammalian channels when these channels are excited by a long preconditioning depolarizing prepulse. The same phenomenon is observed in the case of simultaneous binding of an α-toxin upon site 3 (Cohen *et al.*, 2007). As such, it can be seen that the presence of depressant β-toxins in the scorpion venom may still significantly contribute to the toxicity towards mammals.

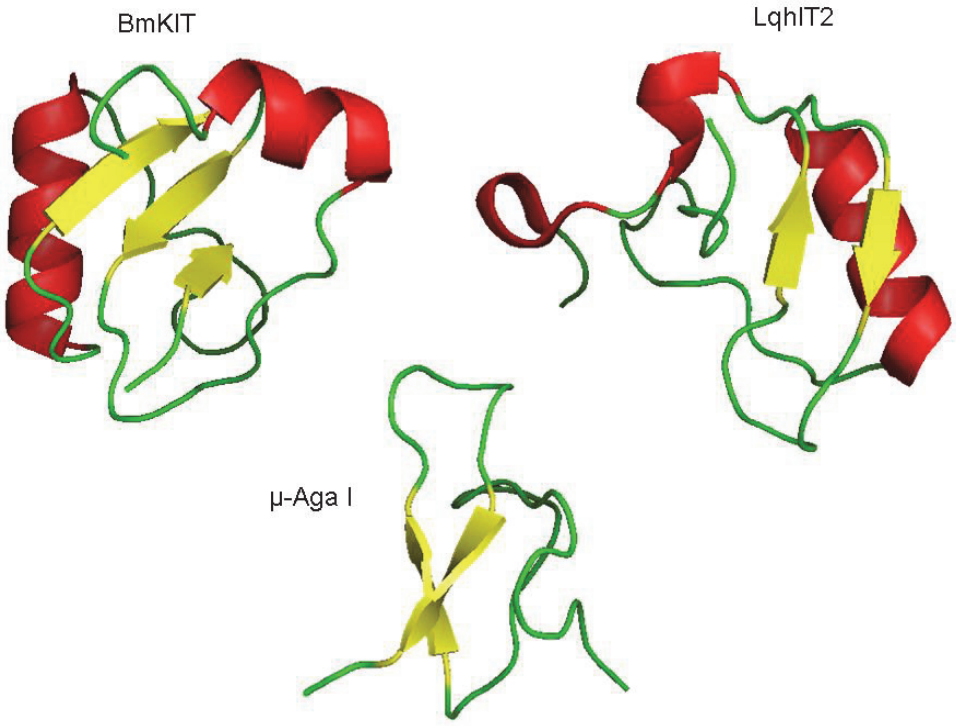


Fig. 13. Structural models of site 4 toxin.

Insect-selective spider and scorpion toxins, represented by μ-Aga1, BmKIT (excitatory toxin) and lqhIT2 (depressant toxin), respectively. Nevertheless these toxins are structurally differing from each other, they all do exert their insecticidal activity through binding upon site 4. PDB ID number: 1EIT, 1WWN and 2I61.

6.4 Insect-selective unidentified Na_V channel interactions: novel site toxins?

Although it has been determined for many spider toxins what the exact site of interaction with the insect Na_V channel is, there are still several insect-selective toxins awaiting further

structure-function and electrophysiological characterization. For example, the exact target site of ACTX-Hi:OB4219, a 38 residue long peptide isolated from the venom of the funnel-web spider *Hadronyche infensa* orchid Beach, has not yet been identified. Determination of its NMR structure revealed that this toxin contains 4 disulfide bridges and is folded accordingly the ICK motif with a triple-stranded antiparallel β-sheet (Rosengren *et al.*, 2002). Interestingly, the cysteine pattern and loop sizes of ACTX-Hi:OB4219 are identical to the μ-agatoxins and to other site 4 spider toxins such as the curtatoxins and δ-palutoxins. However, despite the identical fold is the sequence similarity between ACTX-Hi:OB4219 and these toxins very low. Therefore are electrophysiological and radioligand binding studies required to determine if ACTX-Hi:OB4219 is exerting its insecticidal activity by acting on Na_V channels and if so to identify the exact interaction site with the channel.

Fig. 13. Insect-selective toxins isolated from spider venom binding on unknown sites. Comparison of the amino acid sequences of insect-selective spider toxins which are still awaiting the designation of their binding site. Identical residues are boxed in grey, cysteines are shown in red. The disulfide-bonding pattern is indicated for ACTX-Hi: 0B4219.

DTX9.2, 10 and 11 constitute a family of 56-61 residue long insecticidal peptides, isolated from the venom of the primitive weaving spider *Diguentia canities* (Krapcho *et al.*, 1995). In vivo studies have shown that these toxins do not affect mice when injected intraperitoneal or intracerebroventricular. Because they do cause progressive spastic paralysis in tobacco budworms, can these spider toxins be considered as potential novel insecticides. DTX9.2 caused depolarizations of cockroach axons and repetitive potential discharges in housefly larvae neuromuscular and sensory nerve preparations. TTX blocked these actions, suggesting the involvement of Na_V channel in the mechanism of action of DTX9.2 (Bloomquist *et al.*, 1996). Radioligand binding studies have indicated that it is unlikely that DTX9.2 interacts with site 3 and thus are further structure-function studies necessary to identify the precise target of these interesting insect-selective toxins (Nicholson, 2007).

An ICK motif possessing peptide of 69 residues named Magi 6, from *Macrothele gigas,* induces flaccid paralysis in insects with an even higher potency than the earlier described site 3 toxin Magi 2. However, in contrast to Magi 2, does the site of action for Magi 6 remain unknown. Competitive binding studies indicated that Magi 6 does not compete for sites 3, 4 or 6, neither on insect or mammalian Na_V channels (Corzo *et al.*, 2003). The rapid and strong lethality of Magi 6 when injected in insects, is suggestive of a strong antagonist action on the insect nervous system. Therefore it seems that Magi 6 targets a, for now, unknown site on Na_V channels. Further structure-function studies are awaiting to determine if this is true for Magi 6 or that this toxin is rather interacting with another receptor.

7. The application of insect-selective toxins in the development of novel insecticides

There are 2 main issues that complicate further intensive use of classical industrial pesticides such as DDT and pyrethroids: firstly the growing public awareness of the health hazards and environmental damage caused by conventional agrochemical pesticides has resulted in a general public condemnation regarding the use of these agents (Dong, 2007; Hassan *et al.*, 1990). Secondly, the widespread use of the classical pesticides, together with their limited number of nervous system targets, has resulted in a far-reaching resistance among arthropod populations responsible for the transmission of many human diseases or among pest species involved in major devastation of crops (Nicholson, 2007; Zlotkin *et al.*, 2000). Together, both issues have led to a situation were the current demand of new pesticides is focusing on the one hand on insect-selective agents, minimizing the risk for human health and, on the other hand on potent agents, minimizing the influence on the environment.
Fulfilling this urgent need for new, potent and insect-selective insecticides by insecticidal neurotoxins derived from venomous animals implicates the requirement of formulations which provide these peptide toxins to reach their targets within the circulatory system of insects.
In nature, spiders bite and scorpions and sea anemones sting to deliver the venom directly in the bloodstream of their prey or offender, allowing the neurotoxins to get in direct contact with the Na_V channels. Peptide toxins and polypeptides in general do not penetrate the insect gut and are little to not at all resistant against the proteolytic environment of the insect digestive system. As such does the industrial use of these neurotoxins as insecticidal agents only has prospects depending on the development of means to guarantee a sufficient delivery of peptide toxins to their targets. Therefore a lot of effort has been done to improve the bioavailability of peptides in general and insecticidal peptide toxins specifically. Several strategies are used to overcome the difficulties associated with peptide bioavailability and thus improve the insecticidal potency of neurotoxins.
To implement the insecticidal capacity of polypeptides the following toxin modifications have been proven to increase the bioavailability: (i) Incorporation of the toxin in balcoviruses; (ii) Incorporation of toxins in plants; (iii) Chemical approaches to increase toxin stability and resistance against proteolysis.

7.1 Incorporation of insect-selective toxins in recombinant baculoviruses: biopesticides

One approach to develop environmentally friendly measures to face the highly resistant insect species is the release of baculoviruses that have been genetically engineered to express insecticidal neurotoxins. Exactly because of their safety and insect specificity are insect baculoviruses of great interest as enhancer of neurotoxin bioavailability. The infectivity of baculoviruses is limited to a few closely related species within a single insect family, favoring these viruses as environmental safe insecticides. Their great advantage arises from the fact that baculoviruses do not replace the natural predators, as is the case with chemical insecticides, but rather complements these predators (Zlotkin *et al.*, 2000). From an evolutionary point of view have these viruses adapted themselves for self-preservation and propagation (Zlotkin, 1999). These baculoviruses have developed a strategy as such to keep their host alive as long as possible allowing a maximal progeny production (Bloomquist *et al.*, 1996). This strategy of acting as slow as possible, and in the

mean time permitting the host insect larvae to feed continuously on the crops, is of course a major drawback in an efficient pesticide employment. However, this drawback has been overcome by genetically engineering of baculoviruses to potentiate their insecticidal activity and efficacy. The improved efficacy results from a significant reduced time that the viruses allow their host to feed itself (Bonning & Hammock, 1996). This enhancement of the insecticidal activity is achieved by engineering nucleopolyhedroviruses in such a way that their natural insect pathogenicity is combined with the expression of insect-selective neurotoxins (Gershburg *et al*., 1998). As such a 30-40% improved insecticidal activity is achieved (Chejanovsky *et al*., 1995).

The gene encoding for a insect-selective neurotoxin is subcloned into a transfer vector plasmid, which is then cotransfected with the parental baculovirus DNA into an appropriate insect cell line so that the neurotoxin encoding gene is transferred to the baculovirus by homologous recombination (Zlotkin *et al*., 2000). Insect-selective neurotoxins from spiders, scorpions and sea anemones have been used to the construction of recombinant baculoviruses. Nevertheless, currently there is a limited use of recombinant baculoviruses expressing insect-selective neurotoxins mainly due the lack of well-characterized toxins to be selected from as potential candidates for incorporation into the baculovirus genome. Therefore it is still essential to explore the large pharmacological libraries contained within various venomous animals (Nicholson, 2007).

Even though the insecticidal efficacy of sea anemone and scorpion toxins is higher compared to spider toxins, it should be noted that **spider** Na_V channel toxins do posses some structural features which favors them as candidates for recombinant expression. Spider toxins are smaller in size and because of the smaller number of disulfide bridges, are they more likely to fold correctly. Furthermore, all characterized insect-selective spider toxins up to date are composed of the ICK motif. This conserved scaffold provides spider toxins a highly chemical stability and a strong resistance to a denaturizing and proteolytic environment (Nicholson, 2007; Norton & Pallaghy, 1998). Three spider toxins active on insect Na_V channels have been successfully incorporated into baculovirus genome: μ-Aga IV from *Agelenopsis aperta*, DTX9.2 from *Diguentia canities* and Ta1TX-1 from the hobo spider *Tegenaria agrestis* (Hughes *et al*., 1997; Tomalski *et al*., 1989). Genes encoding for DTX9.2 or Ta1TX-1 were inserted into the *Autographa californica* multiple nuclear polyhedrosis baculovirus (AcMNPV) (Krapcho *et al*., 1995). AcMNPV is a baculovirus of wide interest as it is known to infect a variation of important lepidopterus pests (McCutchen *et al*., 1991). The efficacy of the recombinant baculoviruses expressing these insect selective spider toxins were evaluated in three important lepidopteran insect pests, *Trichoplusia* ni Hubner, *Spodoptera exigua* and *Heliothis virescens.* It was demonstrated that both DTX9.2 and Ta1TX-1 expressing baculoviruses reduced the host feeding time up to 40% and caused a reduction in host survival time ranging from 18% to 25%. Interestingly, the DTX9.2 expressing virus stopped the feeding faster while the Ta1TX-1 virus killed the hosts faster, suggesting that DTX9.2 is more promising in effective reducing crop damage (Hughes *et al*., 1997).

Similar results were obtained when the site 4 spider toxin, μ-Aga IV was expressed by AcMNPV. Infection of lepidopteran larvae with these recombinant baculoviruses resulted in a dramatic improvement of insecticidal efficacy, increasing killing time up to 50% (Prikhod'ko & Miller, 1996). The first insect-specific toxin gene to be transferred into a baculovirus was the highly insecticidal excitatory **scorpion** β-toxin AaIT (*Androctonus australis* Hector) (Stewart *et al*., 1991). Lepidopterous larvae when infected with an AaIT expressing recombinant baculovirus such as BmNPV-AaIT, have a strongly reduced

survival time and hence damage a significant lower amount of host plants compared to wild type viruses. The expressed insect-selective neurotoxin found in the circulation of the infected insects is characterized by the following important properties: (i) Chemical identity with native toxin; (ii) The expressed toxin causes the same physiological symptoms in vivo as the injected native one. In the case of AaIT this is the typical contractile paralysis observed in insects injected by excitatory insect-selective β-toxin. (iii) Expressed neurotoxins display an enhanced affectivity, designated as toxin potentiation and depicted by the fact that the expressed toxin requires a much lower hemolymph titer than the injected native one to cause similar paralysis (Maeda, 1989). It was assumed that this potentiation was the obvious consequence of the insecticidal pharmacokinetic-targeting cooperativity of both the recombinant baculovirus and its expressed scorpion toxin AaIT. However, later studies have indicated that the toxin potentiation observed for expressed toxins is the outcome of two phenomena. Firstly, the continuously toxin expression by the virus infected tracheal epithelia, ramifying within the insect and as thus creating a baculovirus conduit, hereby constituting a pursuing overall supply of newly generated toxin to its specific target namely the insect Na_V channels. Secondly, the translocation of the toxin gene into the central nervous system of the insects provides the expressed toxin a free pathway to the target site which is inaccessible to the native toxin (Zlotkin *et al*., 2000).

Several other insect-selective scorpion toxin have also been field-applicated in combination with baculoviruses. A comparative study was conducted in which the insecticidal efficacy of recombinant expressed depressant β-toxins was paralleled with this of excitatory β-toxins. This study revealed that the depressant toxin LqhIT2 displayed a higher insecticidal activity compared to its excitatory counterpart LqhIT1 (Gershburg *et al*., 1998). The observed difference in activity between these toxins, isolated from the scorpion *Leiurus quinquestriatus hebraeus,* was surprising as excitatory β-toxins generate an immediate paralyzing effect whereas depressant β-toxins generate a delayed effect. The difference might be explained by pharmacokinetic differences resulting from a higher stability and thus increased bioavailability of depressant compared to excitatory β-toxins (Gershburg *et al*., 1998).

Two insect-selective **sea anemone** toxins targeting Na_V channels have been used in the recombinant baculovirus strategy. The site 3 toxins As II, from the sea anemone *Anemonia sulcata* and Sh I isolated from *Stichodactyla helianthus*, displayed an effective ability to reduce crop damage upon expression by AcMNPV (Prikhod'ko & Miller, 1996). Furthermore, the construction of AcMNPV expressing both the site 3 toxin As II and the site 4 toxin μ-Aga-IV, resulted in a synergistic enhanced insecticidal activity, underlining the potentiating of insecticidal activity by coproduction of toxins targeting allostericaly coupled sites of Na_V channels (Nicholson, 2007).

7.2 Incorporation of insect-selective toxins in plants: transgenic plants

Another strategy in the development of potent insect-selective insecticides without burden for the environment is the incorporation of neurotoxins in the genome of plants.

It has been shown that the **spider** toxin ω-ACTX-Hv1a, from *Hadronyche versuta*, displayed remarkable insecticidal activity upon incorporation into the tobacco plant *Nicotiana tabacum* (Fletcher *et al*., 1997). Although ω-ACTX-Hv1a is an insect-selective Ca_V and not a Na_V channel toxin, the insecticidal efficacy of ω-ACTX-Hv1a fusion proteins against important pest species of transgenic plants expressing this insect-selective spider toxin is a strong argument evidencing the efficiency of the transgenic approach. Nevertheless, no insect-selective spider neurotoxins targeting Na_V channels have been selected to be incorporated in

plants (Nicholson, 2007). However, the insect-selective spider toxin Magi 6, isolated from the Japanese spider *Macrothele gigas*, has been successfully expressed in transgenic tobacco. As described earlier is the exact target of this toxin still unknown, although its potent effects *in vivo* do suggest that Magi 6 is acting on a receptor within the nervous system (Corzo *et al.*, 2003). The incorporation of Magi 6 in transgenic plants conferred resistance to several insect pests and opens the possibility of employing this spider toxin to improve the resistance of diverse plants (Hernandez-Campuzano *et al.*, 2009).

Several **scorpion** toxins active on Na_V channels are successfully used as insecticide by application of the transgenic approach. A gene encoding for the high insecticidal scorpion toxin BmKIT from *Buthus martensi Karsch* was, in combination with a gene encoding for a insect-specific chinitase, from *Manduca sexta*, introduced into *Brassica napus* plants rendering these plants resistance against agricultural important pests (Wang *et al.*, 2005). Chitin is a major component is of the cuticle and gut epidermis of many lepidoptera. The chitinase gene is normally not expressed in insects during feeding periods but only in a narrow period prior to molting (Kramer *et al.*, 1993). Continuously exposure to chitinase might lead to malfunction because of chitin degradation and loss of structural integrity of the gut epidermis (Regev *et al.*, 1996). The loss of this absorption barrier in the gut may also enhance the bioavailability of the co expressed neurotoxin as the access pathway for these toxins to reach their targets is strongly facilitated (Wang *et al.*, 2005).

7.3 Improved toxin stability and resistance equals improved activity?

A general criticism upon peptide-based insecticides might be the fact that these Na_V channel targeting toxins despite their extraordinary insecticidal properties potentially suffer from generic problems encountered by all peptide-based drugs, such as short biological half-lives, proteolysis and poor absorption. However, in the last decade there has been an impressive number of studies focusing on the re-engineering of peptide toxins to address these biopharmaceutical problems (Halai & Craik, 2009). Even though the aim of application is different between clinical used drugs and insecticides, the properties of their general pharmacokinetic pathways are identical. As such can techniques used to improve pharmacological availability of peptide-based drugs be very useful to enhance the bioavailability of peptide-based insecticides.

One example of a very effective strategy to improve the drug-like properties of toxins is backbone cyclization, by either the addition of a linker to span the peptide termini or directly by head-to-tail linkage. The resulting cyclic peptides may have an improved binding efficacy but more over display an impressive decrease in susceptibility to proteolysis (Clark *et al.*, 2010). Many microorganisms are known to produce cyclic peptides, exemplified by the well known fungal product cyclosporine which has revolutionized organ transplant therapy because of its potent immunosuppressant activities (Clark *et al.*, 2010; Starzl, 1981). Furthermore, peptide cyclization is a widely used technique in the pharmaceutical industry in the development of a variety of drug design applications (Craik & Adams, 2007). For example, the conotoxin MII, isolated from the *Conus magus*, targets with high potency nicotine acetylcholine receptors (Shon *et al.*, 1997)). Cyclyzation of this toxin by adding a linker of seven amino acids resulted in maintenance of the structure of the native peptide and similar biological activity, but with an importantly enhanced resistance against proteolytic breakdown (Clark *et al.*, 2005). Cyclic peptides used in pharmaceutical applications are usual smaller than 12 amino acids which is noticeable shorter than most Na_V channel targeting neurotoxins described in this review. However, a large number of

naturally occurring disulfide-rich cyclic peptides have been discovered in plants and animals and all of these natural cyclic peptides share the remarkable feature of displaying an exceptional stability and a compact structure (Clark *et al.*, 2010). This suggests that cyclization of highly insecticidal neurotoxins from spiders, scorpions and sea anemone might be potentially useful to enhance their bioavailability by strongly increasing their stability and their resistance against proteolytic degradation.

For peptide toxins and in general for all proteins, several amino acids are sensitive for chemical degradation. Therefore can potential stability problems with peptide-based insecticides be avoided by replacing these residues, provided of course that substituting these residues does not lead to a loss of biological activity (Craik & Adams, 2007). Examples of amino acids susceptible to chemical degradation are deamidation of aspargine, oxidation of methionine and isomerization or cleavage of aspargine-proline bonds (Wakankar & Borchardt, 2006).

A widely used technique in the peptide chemistry is the substitution of $_{L}$-amino acids for $_{D}$-amino acids to improve the resistance against proteases. The use of $_{D}$-amino acids is particularly common in peptide-based drug design of cyclic peptides as described above (Schroeder *et al.*, 2004). An interesting approach to improve the activity of the *Conus striatus* toxin SIIIA is the incorporation of non-natural backbone spacers.

The use of flexible spacers such as amino-3-oxypenatnoic and 6-aminohexanoic acids to replace conformational constrained parts of the three disulfide bridges in SIIIA resulted in an enhanced activity (Green *et al.*, 2007). Since SIIIA is a highly potent inhibitor of mammalian Na_V channels and since it is folded accordingly the ICK motif which is shared by many insect-selective toxins, these observations of enhanced activity are of peculiar interest for the development of new insect-selective insecticides which act by inhibiting the sodium conductance such as HNTX-1.

Other approaches to improve bioavailability are the use of lipid tags on selected residues, terminal capping and peptide truncation. Capping involves C-terminal amidation because it is known that amidation leads to a reduced sensitivity to proteolytic breakdown by carboxypeptidases. Peptide truncation finds it application trough the rationale that residues lying outside the defined cystine framework, display an increased susceptibility to proteolytic or chemical degradation due to the fact that these residues are likely to be more flexible (Craik & Adams, 2007).

All together it can be seen that the increasing knowledge on bypassing the generic barriers insures that the exquisite potency and selectivity of insecticidal peptide toxins derived from venomous animals can be exploited as promising lead compounds in the development of novel peptide-based pesticides.

8. Conclusions and further directions

Arthropods are the most widespread and diverse group of animals worldwide. Even though only a small percentage of all arthropods are considered as pest species, there impact on human society is of great importance. Vector-borne human and veterinary diseases, in which pest species function as vectors for transmission, are of increasing concern to the general population and more specific to the public health. They represent a significant threat to the productivity, health, the normal lifecycle of humans, livestock, companion animals and wild life, and by generalization, to the viability of live on earth (Nicholson, 2007).

Pest species are responsible for major devastations of crops, destroying up to 20 % of the annual crop production worldwide (Nicholson, 2007). An increasing demand on crop production to feed the ever increasing world population, together with the fact that the majority of the world fertile land is being exploited raises a global insurmountable awareness for the urgent need of environmental compatible insecticides, capable of increasing crop production yields by decreasing the losses accountable by pest species. The increasing factors limiting the efficacy of conventional agrochemical pesticides are therefore worrying. Due to the wide usages and the narrow target range of classical agrichemicals, such as DDT and pyrethroids, arthropods have been submitted to a high degree of selection pressure, resulting in pest species with a far reaching resistance against these agents (Brogdon & McAllister, 1998). Consequently, the development of novel, potent and selective pesticides has become crucial for global welfare. Insect-selective neurotoxins might significantly contribute to the accomplishment of novel insecticides.

The range of organisms that is producing toxins for defense or for capturing their arthropod prey is remarkable diverse. Toxins isolated from the venoms of spiders, scorpions and sea anemones, as described in this review, are of particular interest because of their importantly high selectivity for insects over mammals, great potency and environmental friendly character. Spiders, scorpions and sea anemones have developed during their evolution a unique, complex pre-optimized combinatorial peptide library of neurotoxins which is now available as a valuable source of novel insecticides.

Voltage-gated sodium channels have gained great interest as target for future pesticides. This is mainly because of their important role in excitability, their pharmacological diversity exhibited in the large number of binding sites and their pharmacological distinctiveness as revealed by the ability of insect-selective neurotoxins to discriminate strongly between insect and mammalian Na_V channels (Zlotkin, 1999). A very important argument, favoring both neurotoxins and Na_V channels as key elements in the development of new insecticides is the existence of allostericly coupled sites as potentiating of the insecticidal activity. For instance, it has been shown that pyrethroids and scorpion toxins can operate synergistic with one another (Gilles *et al.*, 2003). It was found that insects, which were first infected with a baculovirus expressing the scorpion toxin AahIT, were much more sensitive to the effects of pyrethroids then non-infected species (Sharkey *et al.*, 1987). Interesting to note is the fact that pyrethroid-resistant strains do display an enhanced sensitivity towards AahIT and other site 4 toxins (Zlotkin, 1999). Furthermore it was also demonstrated that site 2, 3 and 5 toxins could enhance pyrethroid binding to locust neuronal membranes (Gilles *et al.*, 2003). Using the toxin ATX II as a site 3 toxin representative, it was shown that sea anemone toxins could similarly enhance the potency of DDT and other pyrethriods (Bloomquist & Soderlund, 1988). All together these results do imply the interesting possibility to use the synergistic interaction of insect-selective Na_V channel toxins in order to not only overcome resistance but also to reduce the concentration of each toxin required to obtain an efficient control of pest species.

The synergistic action of insecticides should be seen beyond the boundaries of the Na_V channel toxins. Their exist many other interesting targets for insecticides such as Ca_V, K_V, GABA-gated Cl channels or other receptors such as nicotine acetylcholine receptors. Furthermore, baculoviruses, snowdrop lectine and chitinases have proven to be very useful in the battle against pest species whether this is because of their direct insecticidal properties or because they function as ideal transport vector for insect-selective toxins. It is recommendable to develop over the long term a strategy of applying synergistic mixtures

containing a variety of insecticidal substances acting on a broad range of different targets. The synergistic character of these mixtures will be the result of positive cooperativity between insecticidal agents acting on different targets and/or agents acting on allosterically coupled sites within the same target. Employment of such mixtures will significantly reduce the required doses of each insecticide and herewith not only their production cost but also their impact on the environment and public health.
Insect-selective Na_V channel toxins from spiders, scorpion and sea anemones can contribute importantly to these synergistic mixtures. They deserve to be lead compounds in the development of new insecticides thanks to the exquisite selectivity, unseen affinity and high potency displayed towards their target, the insect voltage-gated sodium channel.

9. Acknowledgements

The authors are greatly indebted to Annelies Van Der Haegen for help in preparation of the manuscript.

10. References

Adams, M.E. (2004). Agatoxins: ion channel specific toxins from the American funnel web spider, Agelenopsis aperta. *Toxicon*, Vol.43, No.5, (Apr), pp. 509-25

Arnon, T.; Potikha, T.; Sher, D.; Elazar, M.; Mao, W.; Tal, T.; Bosmans, F.; Tytgat, J.; Ben-Arie, N. & Zlotkin, E. (2005). BjalphaIT: a novel scorpion alpha-toxin selective for insects--unique pharmacological tool. *Insect Biochem Mol Biol*, Vol.35, No.3, (Mar), pp. 187-95

Beress, L. & Beress, R. (1975). Purification of three polypeptides with neuro- and cardiotoxic activity from the sea anemone Anemonia sulcata. *Toxicon*, Vol.13, No.5, (Nov), pp. 359-67

Billen, B.; Bosmans, F. & Tytgat, J. (2008). Animal peptides targeting voltage-activated sodium channels. *Curr Pharm Des*, Vol.14, No.24, pp. 2492-502

Billen, B.; Vassilevski, A.; Nikolsky, A.; Debaveye, S.; Tytgat, J. & Grishin, E. (2010). Unique bell-shaped voltage-dependent modulation of Na+ channel gating by novel insect-selective toxins from the spider Agelena orientalis. *J Biol Chem*, Vol.285, No.24, (Jun 11), pp. 18545-54

Bloomquist, J.R.; Kinne, L.P.; Deutsch, V. & Simpson, S.F. (1996). Mode of action of an insecticidal peptide toxin from the venom of a weaving spider (Diguetia canities). *Toxicon*, Vol.34, No.9, (Sep), pp. 1072-5

Bloomquist, J.R. & Soderlund, D.M. (1988). Pyrethroid insecticides and DDT modify alkaloid-dependent sodium channel activation and its enhancement by sea anemone toxin. *Mol Pharmacol*, Vol.33, No.5, (May), pp. 543-50

Bonning, B.C. & Hammock, B.D. (1996). Development of recombinant baculoviruses for insect control. *Annu Rev Entomol*, Vol.41, pp. 191-210

Boore, J.L.; Lavrov, D.V. & Brown, W.M. (1998). Gene translocation links insects and crustaceans. *Nature*, Vol.392, No.6677, (Apr 16), pp. 667-8

Borges, M.H.; De Lima, M.E.; Stankiewicz, M.; Pelhate, M.; Cordeiro, M.N. & Beirão, P.S.L. (2009). Structural and functional diversity in the venom of spiders of the genus *Phoneutria*, In: *Animal toxins: state of the art*, De Lima, M.E., pp. 291-312, EDITORAufmg, 978-85-7041-735-0, Belo Horizonte

Bosmans, F.; Aneiros, A. & Tytgat, J. (2002). The sea anemone Bunodosoma granulifera contains surprisingly efficacious and potent insect-selective toxins. *FEBS Lett*, Vol.532, No.1-2, (Dec 4), pp. 131-4

Bosmans, F.; Martin-Eauclaire, M.F. & Tytgat, J. (2005). The depressant scorpion neurotoxin LqqIT2 selectively modulates the insect voltage-gated sodium channel. *Toxicon*, Vol.45, No.4, (Mar 15), pp. 501-7

Bosmans, F. & Tytgat, J. (2007a). Sea anemone venom as a source of insecticidal peptides acting on voltage-gated Na+ channels. *Toxicon*, Vol.49, No.4, (Mar 15), pp. 550-60

Bosmans, F. & Tytgat, J. (2007b). Voltage-gated sodium channel modulation by scorpion alpha-toxins. *Toxicon*, Vol.49, No.2, (Feb), pp. 142-58

Brogdon, W.G. & McAllister, J.C. (1998). Insecticide resistance and vector control. *Emerg Infect Dis*, Vol.4, No.4, (Oct-Dec), pp. 605-13

Bruce, C.; Fitches, E.C.; Chougule, N.; Bell, H.A. & Gatehouse, J.A. (2011). Recombinant conotoxin, TxVIA, produced in yeast has insecticidal activity. *Toxicon*, Vol.58, No.1, (Jul), pp. 93-100

Bulet, P. & Stocklin, R. (2005). Insect antimicrobial peptides: structures, properties and gene regulation. *Protein Pept Lett*, Vol.12, No.1, (Jan), pp. 3-11

Catterall, W.A. (2000). From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels. *Neuron*, Vol.26, No.1, (Apr), pp. 13-25

Catterall, W.A.; Cestele, S.; Yarov-Yarovoy, V.; Yu, F.H.; Konoki, K. & Scheuer, T. (2007). Voltage-gated ion channels and gating modifier toxins. *Toxicon*, Vol.49, No.2, (Feb), pp. 124-41

Cestele, S.; Scheuer, T.; Mantegazza, M.; Rochat, H. & Catterall, W.A. (2001). Neutralization of gating charges in domain II of the sodium channel alpha subunit enhances voltage-sensor trapping by a beta-scorpion toxin. *J Gen Physiol*, Vol.118, No.3, (Sep), pp. 291-302

Cestele, S.; Yarov-Yarovoy, V.; Qu, Y.; Sampieri, F.; Scheuer, T. & Catterall, W.A. (2006). Structure and function of the voltage sensor of sodium channels probed by a beta-scorpion toxin. *J Biol Chem*, Vol.281, No.30, (Jul 28), pp. 21332-44

Chanda, B. & Bezanilla, F. (2002). Tracking voltage-dependent conformational changes in skeletal muscle sodium channel during activation. *J Gen Physiol*, Vol.120, No.5, (Nov), pp. 629-45

Chejanovsky, N.; Zilberberg, N.; Rivkin, H.; Zlotkin, E. & Gurevitz, M. (1995). Functional expression of an alpha anti-insect scorpion neurotoxin in insect cells and lepidopterous larvae. *FEBS Lett*, Vol.376, No.3, (Dec 4), pp. 181-4

Clark, R.J.; Akcan, M.; Kaas, Q.; Daly, N.L. & Craik, D.J. (2010). Cyclization of conotoxins to improve their biopharmaceutical properties. *Toxicon*, (Dec 10), pp.

Clark, R.J.; Fischer, H.; Dempster, L.; Daly, N.L.; Rosengren, K.J.; Nevin, S.T.; Meunier, F.A.; Adams, D.J. & Craik, D.J. (2005). Engineering stable peptide toxins by means of backbone cyclization: stabilization of the alpha-conotoxin MII. *Proc Natl Acad Sci U S A*, Vol.102, No.39, (Sep 27), pp. 13767-72

Cohen, L.; Gilles, N.; Karbat, I.; Ilan, N.; Gordon, D. & Gurevitz, M. (2006). Direct evidence that receptor site-4 of sodium channel gating modifiers is not dipped in the phospholipid bilayer of neuronal membranes. *J Biol Chem*, Vol.281, No.30, (Jul 28), pp. 20673-9

Cohen, L.; Troub, Y.; Turkov, M.; Gilles, N.; Ilan, N.; Benveniste, M.; Gordon, D. & Gurevitz, M. (2007). Mammalian skeletal muscle voltage-gated sodium channels are affected by scorpion depressant "insect-selective" toxins when preconditioned. *Mol Pharmacol*, Vol.72, No.5, (Nov), pp. 1220-7

Corzo, G.; Diego-Garcia, E.; Clement, H.; Peigneur, S.; Odell, G.; Tytgat, J.; Possani, L.D. & Alagon, A. (2008). An insecticidal peptide from the theraposid Brachypelma smithi spider venom reveals common molecular features among spider species from different genera. *Peptides*, Vol.29, No.11, (Nov), pp. 1901-8

Corzo, G.; Escoubas, P.; Stankiewicz, M.; Pelhate, M.; Kristensen, C.P. & Nakajima, T. (2000). Isolation, synthesis and pharmacological characterization of delta-palutoxins IT, novel insecticidal toxins from the spider Paracoelotes luctuosus (Amaurobiidae). *Eur J Biochem*, Vol.267, No.18, (Sep), pp. 5783-95

Corzo, G.; Escoubas, P.; Villegas, E.; Karbat, I.; Gordon, D.; Gurevitz, M.; Nakajima, T. & Gilles, N. (2005). A spider toxin that induces a typical effect of scorpion alpha-toxins but competes with beta-toxins on binding to insect sodium channels. *Biochemistry*, Vol.44, No.5, (Feb 8), pp. 1542-9

Corzo, G.; Gilles, N.; Satake, H.; Villegas, E.; Dai, L.; Nakajima, T. & Haupt, J. (2003). Distinct primary structures of the major peptide toxins from the venom of the spider Macrothele gigas that bind to sites 3 and 4 in the sodium channel. *FEBS Lett*, Vol.547, No.1-3, (Jul 17), pp. 43-50

Craik, D.J. & Adams, D.J. (2007). Chemical modification of conotoxins to improve stability and activity. *ACS Chem Biol*, Vol.2, No.7, (Jul 20), pp. 457-68

Craik, D.J.; Daly, N.L. & Waine, C. (2001). The cystine knot motif in toxins and implications for drug design. *Toxicon*, Vol.39, No.1, (Jan), pp. 43-60

de Dianous, S.; Kopeyan, C.; Bahraoui, E.M. & Rochat, H. (1987). Purification of contracture-inducing insect toxins from Buthinae scorpion venoms by immunoaffinity and high pressure liquid chromatography. *Toxicon*, Vol.25, No.7, pp. 731-41

de la Vega, R.C. & Possani, L.D. (2007). Novel paradigms on scorpion toxins that affects the activating mechanism of sodium channels. *Toxicon*, Vol.49, No.2, (Feb), pp. 171-80

Defaix, A. & Lapied, B. (2005). Role of a novel maintained low-voltage-activated inward current permeable to sodium and calcium in pacemaking of insect neurosecretory neurons. *Invert Neurosci*, Vol.5, No.3-4, (Nov), pp. 135-46

Derst, C.; Walther, C.; Veh, R.W.; Wicher, D. & Heinemann, S.H. (2006). Four novel sequences in Drosophila melanogaster homologous to the auxiliary Para sodium channel subunit TipE. *Biochem Biophys Res Commun*, Vol.339, No.3, (Jan 20), pp. 939-48

Diochot, S.; Baron, A.; Rash, L.D.; Deval, E.; Escoubas, P.; Scarzello, S.; Salinas, M. & Lazdunski, M. (2004). A new sea anemone peptide, APETx2, inhibits ASIC3, a major acid-sensitive channel in sensory neurons. *Embo J*, Vol.23, No.7, (Apr 7), pp. 1516-25

Diochot, S.; Loret, E.; Bruhn, T.; Beress, L. & Lazdunski, M. (2003). APETx1, a new toxin from the sea anemone Anthopleura elegantissima, blocks voltage-gated human ether-a-go-go-related gene potassium channels. *Mol Pharmacol*, Vol.64, No.1, (Jul), pp. 59-69

Dong, K. (1997). A single amino acid change in the para sodium channel protein is associated with knockdown-resistance (kdr) to pyrethroid insecticides in German cockroach. *Insect Biochem Mol Biol*, Vol.27, No.2, (Feb), pp. 93-100

Dong, K. (2007). Insect sodium channels and insecticide resistance. *Invert Neurosci*, Vol.7, No.1, (Mar), pp. 17-30

Du, Y.; Nomura, Y.; Liu, Z.; Huang, Z.Y. & Dong, K. (2009). Functional expression of an arachnid sodium channel reveals residues responsible for tetrodotoxin resistance in invertebrate sodium channels. *J Biol Chem*, Vol.284, No.49, (Dec 4), pp. 33869-75

Feng, G.; Deak, P.; Chopra, M. & Hall, L.M. (1995). Cloning and functional analysis of TipE, a novel membrane protein that enhances Drosophila para sodium channel function. *Cell*, Vol.82, No.6, (Sep 22), pp. 1001-11

Ferrat, G.; Bosmans, F.; Tytgat, J.; Pimentel, C.; Chagot, B.; Gilles, N.; Nakajima, T.; Darbon, H. & Corzo, G. (2005). Solution structure of two insect-specific spider toxins and their pharmacological interaction with the insect voltage-gated Na+ channel. *Proteins*, Vol.59, No.2, (May 1), pp. 368-79

Fletcher, J.I.; Smith, R.; O'Donoghue, S.I.; Nilges, M.; Connor, M.; Howden, M.E.; Christie, M.J. & King, G.F. (1997). The structure of a novel insecticidal neurotoxin, omega-atracotoxin-HV1, from the venom of an Australian funnel web spider. *Nat Struct Biol*, Vol.4, No.7, (Jul), pp. 559-66

Froy, O.; Zilberberg, N.; Gordon, D.; Turkov, M.; Gilles, N.; Stankiewicz, M.; Pelhate, M.; Loret, E.; Oren, D.A.; Shaanan, B. & Gurevitz, M. (1999). The putative bioactive surface of insect-selective scorpion excitatory neurotoxins. *J Biol Chem*, Vol.274, No.9, (Feb 26), pp. 5769-76

Gershburg, E.; Stockholm, D.; Froy, O.; Rashi, S.; Gurevitz, M. & Chejanovsky, N. (1998). Baculovirus-mediated expression of a scorpion depressant toxin improves the insecticidal efficacy achieved with excitatory toxins. *FEBS Lett*, Vol.422, No.2, (Jan 30), pp. 132-6

Gilles, N.; Gurevitz, M. & Gordon, D. (2003). Allosteric interactions among pyrethroid, brevetoxin, and scorpion toxin receptors on insect sodium channels raise an alternative approach for insect control. *FEBS Lett*, Vol.540, No.1-3, (Apr 10), pp. 81-5

Gilles, N.; Harrison, G.; Karbat, I.; Gurevitz, M.; Nicholson, G.M. & Gordon, D. (2002). Variations in receptor site-3 on rat brain and insect sodium channels highlighted by binding of a funnel-web spider delta-atracotoxin. *Eur J Biochem*, Vol.269, No.5, (Mar), pp. 1500-10

Goldin, A.L. (1999). Diversity of mammalian voltage-gated sodium channels. *Ann N Y Acad Sci*, Vol.868, (Apr 30), pp. 38-50

Goldin, A.L. (2001). Resurgence of sodium channel research. *Annu Rev Physiol*, Vol.63, pp. 871-94

Goldin, A.L.; Barchi, R.L.; Caldwell, J.H.; Hofmann, F.; Howe, J.R.; Hunter, J.C.; Kallen, R.G.; Mandel, G.; Meisler, M.H.; Netter, Y.B.; Noda, M.; Tamkun, M.M.; Waxman, S.G.; Wood, J.N. & Catterall, W.A. (2000). Nomenclature of voltage-gated sodium channels. *Neuron*, Vol.28, No.2, (Nov), pp. 365-8

Gordon, D.; Karbat, I.; Ilan, N.; Cohen, L.; Kahn, R.; Gilles, N.; Dong, K.; Stuhmer, W.; Tytgat, J. & Gurevitz, M. (2007). The differential preference of scorpion alpha-toxins

for insect or mammalian sodium channels: implications for improved insect control. *Toxicon*, Vol.49, No.4, (Mar 15), pp. 452-72

Gordon, D.; Martin-Eauclaire, M.F.; Cestele, S.; Kopeyan, C.; Carlier, E.; Khalifa, R.B.; Pelhate, M. & Rochat, H. (1996). Scorpion toxins affecting sodium current inactivation bind to distinct homologous receptor sites on rat brain and insect sodium channels. *J Biol Chem*, Vol.271, No.14, (Apr 5), pp. 8034-45

Goudet, C.; Ferrer, T.; Galan, L.; Artiles, A.; Batista, C.F.; Possani, L.D.; Alvarez, J.; Aneiros, A. & Tytgat, J. (2001). Characterization of two Bunodosoma granulifera toxins active on cardiac sodium channels. *Br J Pharmacol*, Vol.134, No.6, (Nov), pp. 1195-206

Green, B.R.; Catlin, P.; Zhang, M.M.; Fiedler, B.; Bayudan, W.; Morrison, A.; Norton, R.S.; Smith, B.J.; Yoshikami, D.; Olivera, B.M. & Bulaj, G. (2007). Conotoxins containing nonnatural backbone spacers: cladistic-based design, chemical synthesis, and improved analgesic activity. *Chem Biol*, Vol.14, No.4, (Apr), pp. 399-407

Grolleau, F. & Lapied, B. (2000). Dorsal unpaired median neurones in the insect central nervous system: towards a better understanding of the ionic mechanisms underlying spontaneous electrical activity. *J Exp Biol*, Vol.203, No.Pt 11, (Jun), pp. 1633-48

Guan, R.J.; Xiang, Y.; He, X.L.; Wang, C.G.; Wang, M.; Zhang, Y.; Sundberg, E.J. & Wang, D.C. (2004). Structural mechanism governing cis and trans isomeric states and an intramolecular switch for cis/trans isomerization of a non-proline peptide bond observed in crystal structures of scorpion toxins. *J Mol Biol*, Vol.341, No.5, (Aug 27), pp. 1189-204

Gurevitz, M.; Froy, O.; Zilberberg, N.; Turkov, M.; Strugatsky, D.; Gershburg, E.; Lee, D.; Adams, M.E.; Tugarinov, V.; Anglister, J.; Shaanan, B.; Loret, E.; Stankiewicz, M.; Pelhate, M.; Gordon, D. & Chejanovsky, N. (1998). Sodium channel modifiers from scorpion venom: structure-activity relationship, mode of action and application. *Toxicon*, Vol.36, No.11, (Nov), pp. 1671-82

Gurevitz, M.; Karbat, I.; Cohen, L.; Ilan, N.; Kahn, R.; Turkov, M.; Stankiewicz, M.; Stuhmer, W.; Dong, K. & Gordon, D. (2007). The insecticidal potential of scorpion beta-toxins. *Toxicon*, Vol.49, No.4, (Mar 15), pp. 473-89

Halai, R. & Craik, D.J. (2009). Conotoxins: natural product drug leads. *Nat Prod Rep*, Vol.26, No.4, (Apr), pp. 526-36

Hassan, A.A.; el-Sewedy, S.M.; Minatogawa, Y. & Kido, R. (1990). Effects of some insecticides on several enzymes of tryptophan metabolism in rats. *J Environ Sci Health B*, Vol.25, No.3, (Jun), pp. 333-46

Hernandez-Campuzano, B.; Suarez, R.; Lina, L.; Hernandez, V.; Villegas, E.; Corzo, G. & Iturriaga, G. (2009). Expression of a spider venom peptide in transgenic tobacco confers insect resistance. *Toxicon*, Vol.53, No.1, (Jan), pp. 122-8

Hille, B. (2001). *Ion channels of excitable membranes* (3), Sinauer Associates, Inc., 978-0-87893-321-1, Sunderland, Massachusetts

Honma, T. & Shiomi, K. (2006). Peptide toxins in sea anemones: structural and functional aspects. *Mar Biotechnol (NY)*, Vol.8, No.1, (Jan-Feb), pp. 1-10

Hughes, P.R.; Wood, H.A.; Breen, J.P.; Simpson, S.F.; Duggan, A.J. & Dybas, J.A. (1997). Enhanced Bioactivity of Recombinant Baculoviruses Expressing Insect-Specific

Spider Toxins in Lepidopteran Crop Pests. *J Invertebr Pathol*, Vol.69, No.2, (Mar), pp. 112-8

Hwang, D.F.; Tai, K.P.; Chueh, C.H.; Lin, L.C. & Jeng, S.S. (1991). Tetrodotoxin and derivatives in several species of the gastropod Naticidae. *Toxicon*, Vol.29, No.8, pp. 1019-24

Ingles, P.J.; Adams, P.M.; Knipple, D.C. & Soderlund, D.M. (1996). Characterization of voltage-sensitive sodium channel gene coding sequences from insecticide-susceptible and knockdown-resistant house fly strains. *Insect Biochem Mol Biol*, Vol.26, No.4, (Apr), pp. 319-26

Isbister, G.K. & White, J. (2004). Clinical consequences of spider bites: recent advances in our understanding. *Toxicon*, Vol.43, No.5, (Apr), pp. 477-92

Ishida, M.; Yokoyama, A.; Shimakura, K.; Nagashima, Y. & Shiomi, K. (1997). Halcurin, a polypeptide toxin from the sea anemone Halcurias sp., with a structural resemblance to type 1 and 2 toxins. *Toxicon*, Vol.35, No.4, (Apr), pp. 537-44

Jeziorski, M.C.; Greenberg, R.M. & Anderson, P.A. (2000). The molecular biology of invertebrate voltage-gated Ca(2+) channels. *J Exp Biol*, Vol.203, No.Pt 5, (Mar), pp. 841-56

Karbat, I.; Turkov, M.; Cohen, L.; Kahn, R.; Gordon, D.; Gurevitz, M. & Frolow, F. (2007). X-ray structure and mutagenesis of the scorpion depressant toxin LqhIT2 reveals key determinants crucial for activity and anti-insect selectivity. *J Mol Biol*, Vol.366, No.2, (Feb 16), pp. 586-601

King, G.F.; Escoubas, P. & Nicholson, G.M. (2008). Peptide toxins that selectively target insect Na(V) and Ca(V) channels. *Channels (Austin)*, Vol.2, No.2, (Mar-Apr), pp. 100-16

Kramer, K.J.; Corpuz, L.; Choi, H.K. & Muthukrishnan, S. (1993). Sequence of a cDNA and expression of the gene encoding epidermal and gut chitinases of Manduca sexta. *Insect Biochem Mol Biol*, Vol.23, No.6, (Sep), pp. 691-701

Krapcho, K.J.; Kral, R.M., Jr.; Vanwagenen, B.C.; Eppler, K.G. & Morgan, T.K. (1995). Characterization and cloning of insecticidal peptides from the primitive weaving spider Diguetia canities. *Insect Biochem Mol Biol*, Vol.25, No.9, (Oct), pp. 991-1000

Kuhn-Nentwig, L.; Schaller, J. & Nentwig, W. (2004). Biochemistry, toxicology and ecology of the venom of the spider Cupiennius salei (Ctenidae). *Toxicon*, Vol.43, No.5, (Apr), pp. 543-53

Lee, S.H.; Smith, T.J.; Ingles, P.J. & Soderlund, D.M. (2000). Cloning and functional characterization of a putative sodium channel auxiliary subunit gene from the house fly (Musca domestica). *Insect Biochem Mol Biol*, Vol.30, No.6, (Jun), pp. 479-87

Leipold, E.; Hansel, A.; Borges, A. & Heinemann, S.H. (2006). Subtype specificity of scorpion beta-toxin Tz1 interaction with voltage-gated sodium channels is determined by the pore loop of domain 3. *Mol Pharmacol*, Vol.70, No.1, (Jul), pp. 340-7

Li, D.; Xiao, Y.; Hu, W.; Xie, J.; Bosmans, F.; Tytgat, J. & Liang, S. (2003). Function and solution structure of hainantoxin-I, a novel insect sodium channel inhibitor from the Chinese bird spider Selenocosmia hainana. *FEBS Lett*, Vol.555, No.3, (Dec 18), pp. 616-22

Littleton, J.T. & Ganetzky, B. (2000). Ion channels and synaptic organization: analysis of the Drosophila genome. *Neuron*, Vol.26, No.1, (Apr), pp. 35-43

Loret, E.P.; del Valle, R.M.; Mansuelle, P.; Sampieri, F. & Rochat, H. (1994). Positively charged amino acid residues located similarly in sea anemone and scorpion toxins. *J Biol Chem*, Vol.269, No.24, (Jun 17), pp. 16785-8

Loughney, K.; Kreber, R. & Ganetzky, B. (1989). Molecular analysis of the para locus, a sodium channel gene in Drosophila. *Cell*, Vol.58, No.6, (Sep 22), pp. 1143-54

Maeda, S. (1989). Expression of foreign genes in insects using baculovirus vectors. *Annu Rev Entomol*, Vol.34, pp. 351-72

Martin-Eauclaire, M.F.; Ceard, B.; Bosmans, F.; Rosso, J.P.; Tytgat, J. & Bougis, P.E. (2005). New "Birtoxin analogs" from Androctonus australis venom. *Biochem Biophys Res Commun*, Vol.333, No.2, (Jul 29), pp. 524-30

Martin, M.F.; Garcia y Perez, L.G.; el Ayeb, M.; Kopeyan, C.; Bechis, G.; Jover, E. & Rochat, H. (1987). Purification and chemical and biological characterizations of seven toxins from the Mexican scorpion, Centruroides suffusus suffusus. *J Biol Chem*, Vol.262, No.10, (Apr 5), pp. 4452-9

McCutchen, B.F.; Choudary, P.V.; Crenshaw, R.; Maddox, D.; Kamita, S.G.; Palekar, N.; Volrath, S.; Fowler, E.; Hammock, B.D. & Maeda, S. (1991). Development of a recombinant baculovirus expressing an insect-selective neurotoxin: potential for pest control. *Biotechnology (N Y)*, Vol.9, No.9, (Sep), pp. 848-52

Mebs, D. (2002). *Venomous and poisonous animals* (1), medpharm Scientific Publishers, 3-88763-093-9, Stuttgart

Moore, A.W.; Barbel, S.; Jan, L.Y. & Jan, Y.N. (2000). A genomewide survey of basic helix-loop-helix factors in Drosophila. *Proc Natl Acad Sci U S A*, Vol.97, No.19, (Sep 12), pp. 10436-41

Mouhat, S.; Jouirou, B.; Mosbah, A.; De Waard, M. & Sabatier, J.M. (2004). Diversity of folds in animal toxins acting on ion channels. *Biochem J*, Vol.378, No.Pt 3, (Mar 15), pp. 717-26

Narahashi, T. (2008). Tetrodotoxin: a brief history. *Proc Jpn Acad Ser B Phys Biol Sci*, Vol.84, No.5, pp. 147-54

Nardi, F.; Spinsanti, G.; Boore, J.L.; Carapelli, A.; Dallai, R. & Frati, F. (2003). Hexapod origins: monophyletic or paraphyletic? *Science*, Vol.299, No.5614, (Mar 21), pp. 1887-9

Nicholson, G.M. (2007). Insect-selective spider toxins targeting voltage-gated sodium channels. *Toxicon*, Vol.49, No.4, (Mar 15), pp. 490-512

Nicholson, G.M.; Little, M.J. & Birinyi-Strachan, L.C. (2004). Structure and function of delta-atracotoxins: lethal neurotoxins targeting the voltage-gated sodium channel. *Toxicon*, Vol.43, No.5, (Apr), pp. 587-99

Norton, R.S. (1991). Structure and structure-function relationships of sea anemone proteins that interact with the sodium channel. *Toxicon*, Vol.29, No.9, pp. 1051-84

Norton, R.S. & Pallaghy, P.K. (1998). The cystine knot structure of ion channel toxins and related polypeptides. *Toxicon*, Vol.36, No.11, (Nov), pp. 1573-83

Oliveira, J.S.; Fuentes-Silva, D. & Zaharenko, A.J. (2009). Sea anemone peptides, In: *Animal toxins: state of the art*, De Lima, M.E., pp. 49-72, EDITORAufmg, 978-85-7041-735-0, Belo Horizonte

Pimenta, A.M. & De Lima, M.E. (2005). Small peptides, big world: biotechnological potential in neglected bioactive peptides from arthropod venoms. *J Pept Sci*, Vol.11, No.11, (Nov), pp. 670-6

Platnick, N.I. (2000). The world spider catalog, version 12.0, In: *The American Museum of Natural History*, July 26th 2011, Available from: http://research.amnh.org/iz/spiders/catalog

Possani, L.D.; Becerril, B.; Delepierre, M. & Tytgat, J. (1999). Scorpion toxins specific for Na+-channels. *Eur J Biochem*, Vol.264, No.2, (Sep), pp. 287-300

Prikhod'ko, E.A. & Miller, L.K. (1996). Induction of apoptosis by baculovirus transactivator IE1. *J Virol*, Vol.70, No.10, (Oct), pp. 7116-24

Regev, A.; Keller, M.; Strizhov, N.; Sneh, B.; Prudovsky, E.; Chet, I.; Ginzberg, I.; Koncz-Kalman, Z.; Koncz, C.; Schell, J. & Zilberstein, A. (1996). Synergistic activity of a Bacillus thuringiensis delta-endotoxin and a bacterial endochitinase against Spodoptera littoralis larvae. *Appl Environ Microbiol*, Vol.62, No.10, (Oct), pp. 3581-6

Rodriguez de la Vega, R.C. & Possani, L.D. (2005). Overview of scorpion toxins specific for Na+ channels and related peptides: biodiversity, structure-function relationships and evolution. *Toxicon*, Vol.46, No.8, (Dec 15), pp. 831-44

Rosengren, K.J.; Wilson, D.; Daly, N.L.; Alewood, P.F. & Craik, D.J. (2002). Solution structures of the cis- and trans-Pro30 isomers of a novel 38-residue toxin from the venom of Hadronyche Infensa sp. that contains a cystine-knot motif within its four disulfide bonds. *Biochemistry*, Vol.41, No.10, (Mar 12), pp. 3294-301

Salceda, E.; Perez-Castells, J.; Lopez-Mendez, B.; Garateix, A.; Salazar, H.; Lopez, O.; Aneiros, A.; Standker, L.; Beress, L.; Forssmann, W.G.; Soto, E.; Jimenez-Barbero, J. & Gimenez-Gallego, G. (2007). CgNa, a type I toxin from the giant Caribbean sea anemone Condylactis gigantea shows structural similarities to both type I and II toxins, as well as distinctive structural and functional properties(1). *Biochem J*, Vol.406, No.1, (Aug 15), pp. 67-76

Salgado, V.L. & Kem, W.R. (1992). Actions of three structurally distinct sea anemone toxins on crustacean and insect sodium channels. *Toxicon*, Vol.30, No.11, (Nov), pp. 1365-81

Schroeder, C.I.; Smythe, M.L. & Lewis, R.J. (2004). Development of small molecules that mimic the binding of omega-conotoxins at the N-type voltage-gated calcium channel. *Mol Divers*, Vol.8, No.2, pp. 127-34

Schweitz, H. (1984). Lethal potency in mice of toxins from scorpion, sea anemone, snake and bee venoms following intraperitoneal and intracisternal injection. *Toxicon*, Vol.22, No.2, pp. 308-11

Sharkey, R.G.; Jover, E.; Couraud, F.; Baden, D.G. & Catterall, W.A. (1987). Allosteric modulation of neurotoxin binding to voltage-sensitive sodium channels by Ptychodiscus brevis toxin 2. *Mol Pharmacol*, Vol.31, No.3, (Mar), pp. 273-8

Shon, K.J.; Grilley, M.; Jacobsen, R.; Cartier, G.E.; Hopkins, C.; Gray, W.R.; Watkins, M.; Hillyard, D.R.; Rivier, J.; Torres, J.; Yoshikami, D. & Olivera, B.M. (1997). A noncompetitive peptide inhibitor of the nicotinic acetylcholine receptor from Conus purpurascens venom. *Biochemistry*, Vol.36, No.31, (Aug 5), pp. 9581-7

Shultz, J.W. & Regier, J.C. (2000). Phylogenetic analysis of arthropods using two nuclear protein-encoding genes supports a crustacean + hexapod clade. *Proc Biol Sci*, Vol.267, No.1447, (May 22), pp. 1011-9

Soderlund, D.M. & Knipple, D.C. (2003). The molecular biology of knockdown resistance to pyrethroid insecticides. *Insect Biochem Mol Biol*, Vol.33, No.6, (Jun), pp. 563-77

Song, W.; Du, Y.; Liu, Z.; Luo, N.; Turkov, M.; Gordon, D.; Gurevitz, M.; Goldin, A.L. & Dong, K. (2011). Substitutions in the domain III voltage-sensing module enhance the sensitivity of an insect sodium channel to a scorpion beta-toxin. *J Biol Chem*, Vol.286, No.18, (May 6), pp. 15781-8

Stapleton, A.; Blankenship, D.T.; Ackermann, B.L.; Chen, T.M.; Gorder, G.W.; Manley, G.D.; Palfreyman, M.G.; Coutant, J.E. & Cardin, A.D. (1990). Curtatoxins. Neurotoxic insecticidal polypeptides isolated from the funnel-web spider Hololena curta. *J Biol Chem*, Vol.265, No.4, (Feb 5), pp. 2054-9

Starzl, T.E. (1981). The succession from kidney to liver transplantation. *Transplant Proc*, Vol.13, No.1 Suppl 1, (Mar), pp. 50-4

Stephan, M.M.; Potts, J.F. & Agnew, W.S. (1994). The microI skeletal muscle sodium channel: mutation E403Q eliminates sensitivity to tetrodotoxin but not to mu-conotoxins GIIIA and GIIIB. *J Membr Biol*, Vol.137, No.1, (Jan), pp. 1-8

Stewart, L.M.; Hirst, M.; Lopez Ferber, M.; Merryweather, A.T.; Cayley, P.J. & Possee, R.D. (1991). Construction of an improved baculovirus insecticide containing an insect-specific toxin gene. *Nature*, Vol.352, No.6330, (Jul 4), pp. 85-8

Strugatsky, D.; Zilberberg, N.; Stankiewicz, M.; Ilan, N.; Turkov, M.; Cohen, L.; Pelhate, M.; Gilles, N.; Gordon, D. & Gurevitz, M. (2005). Genetic polymorphism and expression of a highly potent scorpion depressant toxin enable refinement of the effects on insect Na channels and illuminate the key role of Asn-58. *Biochemistry*, Vol.44, No.25, (Jun 28), pp. 9179-87

Tan, J.; Liu, Z.; Nomura, Y.; Goldin, A.L. & Dong, K. (2002a). Alternative splicing of an insect sodium channel gene generates pharmacologically distinct sodium channels. *J Neurosci*, Vol.22, No.13, (Jul 1), pp. 5300-9

Tan, J.; Liu, Z.; Tsai, T.D.; Valles, S.M.; Goldin, A.L. & Dong, K. (2002b). Novel sodium channel gene mutations in Blattella germanica reduce the sensitivity of expressed channels to deltamethrin. *Insect Biochem Mol Biol*, Vol.32, No.4, (Apr), pp. 445-54

Tomalski, M.D.; Kutney, R.; Bruce, W.A.; Brown, M.R.; Blum, M.S. & Travis, J. (1989). Purification and characterization of insect toxins derived from the mite, Pyemotes tritici. *Toxicon*, Vol.27, No.10, pp. 1151-67

Tugarinov, V.; Kustanovich, I.; Zilberberg, N.; Gurevitz, M. & Anglister, J. (1997). Solution structures of a highly insecticidal recombinant scorpion alpha-toxin and a mutant with increased activity. *Biochemistry*, Vol.36, No.9, (Mar 4), pp. 2414-24

Valdez-Cruz, N.A.; Batista, C.V.; Zamudio, F.Z.; Bosmans, F.; Tytgat, J. & Possani, L.D. (2004). Phaiodotoxin, a novel structural class of insect-toxin isolated from the venom of the Mexican scorpion Anuroctonus phaiodactylus. *Eur J Biochem*, Vol.271, No.23-24, (Dec), pp. 4753-61

Vassilevski, A.A.; Kozlov, S.A. & Grishin, E.V. (2009). Molecular diversity of spider venom. *Biochemistry (Mosc)*, Vol.74, No.13, (Dec), pp. 1505-34

Vijverberg, H.P.; Pauron, D. & Lazdunski, M. (1984). The effect of Tityus serrulatus scorpion toxin gamma on Na channels in neuroblastoma cells. *Pflugers Arch*, Vol.401, No.3, (Jul), pp. 297-303

Wakankar, A.A. & Borchardt, R.T. (2006). Formulation considerations for proteins susceptible to asparagine deamidation and aspartate isomerization. *J Pharm Sci*, Vol.95, No.11, (Nov), pp. 2321-36

Wang, J.; Chen, Z.; Du, J.; Sun, Y. & Liang, A. (2005). Novel insect resistance in Brassica napus developed by transformation of chitinase and scorpion toxin genes. *Plant Cell Rep*, Vol.24, No.9, (Nov), pp. 549-55

Wang, R.; Huang, Z.Y. & Dong, K. (2003). Molecular characterization of an arachnid sodium channel gene from the varroa mite (Varroa destructor). *Insect Biochem Mol Biol*, Vol.33, No.7, (Jul), pp. 733-9

Warmke, J.W.; Reenan, R.A.; Wang, P.; Qian, S.; Arena, J.P.; Wang, J.; Wunderler, D.; Liu, K.; Kaczorowski, G.J.; Van der Ploeg, L.H.; Ganetzky, B. & Cohen, C.J. (1997). Functional expression of Drosophila para sodium channels. Modulation by the membrane protein TipE and toxin pharmacology. *J Gen Physiol*, Vol.110, No.2, (Aug), pp. 119-33

Wilson, M.J.; Zhang, M.M.; Azam, L.; Olivera, B.M.; Bulaj, G. & Yoshikami, D. (2011). NaV{beta} Subunits Modulate the Inhibition of NaV1.8 by the Analgesic Gating Modifier {micro}O-Conotoxin MrVIB. *J Pharmacol Exp Ther*, Vol.338, No.2, (Aug), pp. 687-93

Xiao, Y.; Bingham, J.P.; Zhu, W.; Moczydlowski, E.; Liang, S. & Cummins, T.R. (2008). Tarantula huwentoxin-IV inhibits neuronal sodium channels by binding to receptor site 4 and trapping the domain ii voltage sensor in the closed configuration. *J Biol Chem*, Vol.283, No.40, (Oct 3), pp. 27300-13

Xiao, Y. & Liang, S. (2003). Inhibition of neuronal tetrodotoxin-sensitive Na+ channels by two spider toxins: hainantoxin-III and hainantoxin-IV. *Eur J Pharmacol*, Vol.477, No.1, (Sep 5), pp. 1-7

Yu, F.H. & Catterall, W.A. (2003). Overview of the voltage-gated sodium channel family. *Genome Biol*, Vol.4, No.3, pp. 207

Zamudio, F.Z.; Conde, R.; Arevalo, C.; Becerril, B.; Martin, B.M.; Valdivia, H.H. & Possani, L.D. (1997). The mechanism of inhibition of ryanodine receptor channels by imperatoxin I, a heterodimeric protein from the scorpion Pandinus imperator. *J Biol Chem*, Vol.272, No.18, (May 2), pp. 11886-94

Zhu, S.; Gao, B. & Tytgat, J. (2005). Phylogenetic distribution, functional epitopes and evolution of the CSalphabeta superfamily. *Cell Mol Life Sci*, Vol.62, No.19-20, (Oct), pp. 2257-69

Zlotkin, E. (1999). The insect voltage-gated sodium channel as target of insecticides. *Annu Rev Entomol*, Vol.44, pp. 429-55

Zlotkin, E.; Eitan, M.; Bindokas, V.P.; Adams, M.E.; Moyer, M.; Burkhart, W. & Fowler, E. (1991). Functional duality and structural uniqueness of depressant insect-selective neurotoxins. *Biochemistry*, Vol.30, No.19, (May 14), pp. 4814-21

Zlotkin, E.; Fishman, Y. & Elazar, M. (2000). AaIT: from neurotoxin to insecticide. *Biochimie*, Vol.82, No.9-10, (Sep-Oct), pp. 869-81

Zlotkin, E.; Gurevitz, M.; Fowler, E. & Adams, M.E. (1993). Depressant insect selective neurotoxins from scorpion venom: chemistry, action, and gene cloning. *Arch Insect Biochem Physiol*, Vol.22, No.1-2, pp. 55-73

Zlotkin, E.; Kadouri, D.; Gordon, D.; Pelhate, M.; Martin, M.F. & Rochat, H. (1985). An excitatory and a depressant insect toxin from scorpion venom both affect sodium conductance and possess a common binding site. *Arch Biochem Biophys*, Vol.240, No.2, (Aug 1), pp. 877-87

11

Pyrethroid Insecticides: Use, Environmental Fate, and Ecotoxicology

Katherine Palmquist, Johanna Salatas and Anne Fairbrother
Exponent
United States

1. Introduction

There was little public concern over potential nontarget effects of pesticides until the 1962 publication of Rachel Carson's *Silent Spring* (Delaplane, 2000; Moore et al., 2009). Organochlorine insecticides (such as DDT) had been used intensively and were credited for many of the problems highlighted by Carson. Because of their accumulation in the environment and reproductive toxicity in people, their use was eventually phased out in most industrialized countries in the late 1970s and early 1980s (Moore et al., 2009). Organophosphate (OPs) and carbamate pesticides (based on the same chemistry and mode of action as nerve gas used by the military) took the place of the organochlorine products and have been widely used since the late 1970s.

For the past three decades, OP pesticides have been the insecticides most commonly used by professional pest-control bodies and homeowners (Feo et al., 2010). However, in the late 1980s, OPs in California came under scrutiny when they began to show up in groundwater samples. In addition, because of their potentially toxic effects on people, The U.S. Environmental Protection Agency (EPA) considered a ban on both OP and carbamate insecticides as part of the Food Quality Protection Act (FQPA) of 1996. The FQPA mandated that the EPA reassess all pesticide tolerance levels considering aggregate exposures, cumulative effects from pesticides sharing common mechanisms of toxicity, and special protection for infants and children (Metcalfe et al. 2002). During the early 2000s, the EPA began phasing out residential uses of the two primary OPs, diazinon, and chlorpyrifos (CDPR 2008). EPA's decision to eliminate certain uses of the OP insecticides because of their potential for causing toxicity in people, especially children, has led to their gradual replacement with another class of insecticides, the pyrethroids (Oros & Werner, 2005).

2. Use, chemistry and mode-of action of pyrethroids

2.1 Brief history of pyrethroids

Pyrethroids are synthetic analogues of pyrethrins, insecticidal substances obtained from the flowers of a species of chrysanthemum (*Chrysanthemum cinerariaefolium*). The valuable insecticidal properties of pyrethrum (a mixture of pyrethrins, cinerins, and jasmolins) were recognized in the 19th century, and their properties stimulated detailed examination of the chemical constitution of the active esters in the first quarter of the 20th century (Elliot, 1977; Ecobichon, 1996). Chemical research was conducted initially by British, Japanese and French

scientists to find more stable and effective compounds with a mammalian toxicity as low as that of the model Pyrethrin I (Elliott et al. 1979, Pap et al. 1996). Although the acid moieties of the esters were correctly identified early on, only in 1947 were the structures of the alcohols settled. The first synthetic pyrethroid, allethrin, was synthesized in 1949, and marketed in 1952 for the control of household insects (Elliot, 1977; 1980a)

The first set of synthetic compounds containing chrysanthemic acid showed only moderately higher insecticidal potency than that of Pyrethrin I, but they proved to be potent knockdown agents. Pyrethroids and pyrethrins affect nerve impulse transmission in insects mainly through their action on voltage-sensitive sodium channels (Soderlund & Bloomquist, 1989). Efficacy was increased by changing the alcoholic component to the 5-benzy-3-furylmethyl moiety, resulting in tetramethrin, which was first synthesized in 1964 and marketed in 1965 for indoor pest control; and resmethrin, first produced in 1967 and marketed in 1969 for control of household and public health insects (WHO, 1989a). These were the first synthetic compounds with good knockdown activity, greater insecticidal activity and lower mammalian toxicity than the natural esters (Elliot, 1977). Esterifying (1RS, *cis,trans*)-2,2-dimethyl-3-(2,2-dimethylvinyl)cyclopropanecarboxylic acid (chrysanthemic acid) with 3-phenoxybenzyl alcohol resulted in a relatively cheap molecule and further improved insecticide activity, leading to phenothrin in 1969, which has been in commercial use since 1977 to control insects in the home and for public health, and to protect stored grain (Pap et al., 1996; WHO, 1990a). Halogenation of the vinyl group on the cyclopropane ring led to permethrin, which was synthesized in 1973 and produced the photostability required for field use. Permethrin was marketed beginning in 1977 mostly for agricultural purposes, but also for body lice, mosquito nets and other household uses (Pap et al., 1996; WHO, 1990a). Fenvalerate and deltamethrin came on the market in 1976 and 1977, respectively, for use on cotton and other crops, and also for control of cattle insects. In 1974, the introduction of the alpha-cyano group resulted in cypermethrin, which was initially marketed in 1977 as having exceptionally high efficacy against pests in the household, in agriculture, public health, and animal husbandry (WHO, 1989b; Pap et al, 1996). Many second generation pyrethroids (e.g., bifenthrin, cyfluthrin, lambda cyhalothrin) became available in the 1980s (Weston et al., 2004). Original patents expired in the early 1990s, allowing many other manufacturers to offer new active ingredients by the mid-1990s (Pap et al., 1996).

2.2 Pyrethroid mode of action and chemical properties

Unlike the OP insecticides that act on the central nervous system of exposed animals, pyrethroid insecticides disrupt the normal function of the peripheral nervous system. Pyrethroids react with voltage-gated sodium channels on nerves, prolonging the time during which the channels are open. This results in altered nerve function, which manifests either as a series of short bursts or a prolonged burst, and is caused by repetitive discharge of nerve signals or stimulus-dependent nerve depolarization. In general, exposure to toxic doses of these compounds causes incoordination, convulsions, and paralysis (Soderlund & Bloomquist, 1989). Based on observed symptoms and mode-of-action on sodium channels, pyrethroid insecticides can be further categorized into separate classes, type I and type II. Type I pyrethroids, which include allethrin, bifenthrin, d-phenothrin, permethrin, resmethrin and tetramethrin, cause restlessness, hyperexcitation, prostration, and body tremors; Type I pyrethroids produce repetitive nerve discharges. Conversely, exposure to Type II pyrethroids, such as cyhalothrin, cypermethrin, cyfluthrin, deltamethrin,

esfenvalerate, fenvalerate, and fluvalinate, and lambda-cyhalothrin, results in hyperactivity, incoordination, convulsions and writhing; Type II pyrethroids produce stimulus-dependent nerve depolarization and blockage (Ecobichon, 1996: Soderlund & Bloomquist, 1989). This distinction was primarily derived using mammalian studies, and although exposed insects also exhibit different symptoms between the two types, these are not so distinct (Stenerson, 2004).

Also in contrast to OP insecticides, pyrethroids exhibit low toxicity to mammals and birds, while also demonstrating strong selectivity for insects and invertebrates (Ecobichon, 1996; Fishel, 2005). Chronic animal feeding studies have produced high no-effect levels, indicating both a low potential to bioaccumulate and proficient detoxicification in mammalian receptors. In addition, although these compounds are categorized as highly toxic to very highly toxic to nontarget fish and invertebrate species, they are reportedly practically non-toxic to birds (Fishel, 2005). In addition to low avian and mammalian toxicity, physical properties of pyrethroids also are an improvement over their predecessors. The low vapor pressures and high octanol-water coefficients of pyrethroids indicate a low propensity to volatilize and a high affinity for organic matter, soils and clay (Oros & Werner, 2005). Sorption to particulates reduces bioavailability to nontarget organisms, and also makes it more likely that these insecticides will be retained at the application site.

2.3 Current uses of pyrethroids

Synthetic pyrethroid insecticides have been used for more than 20 years to control insect pests in a variety of crops (Maund et al., 2001), but they have become increasingly popular following outright bans or limitations on the use of cholinesterase-inhibiting insecticides (Luo & Zhang, 2011; Feo et al., 2010). In 2000-2001, EPA withdrew the residential registrations for two commonly applied OP pesticides, chlorpyrifos and diazinon (EPA 2000; 2001), resulting in a significant increase in the market penetration of the pyrethroid products. The pyrethroids have a high share of the insecticide market because their activity profile indicates high efficiency, wide spectrum, low mammalian and avian toxicity and biodegradability (Pap et al., 1996). Today, pyrethroids are used in agriculture, forestry, horticulture, public health (i.e., hospitals) and are active ingredients of many insect-control products intended for indoor home use (Feo et al., 2010).

Pyrethrins and synthetic pyrethroids are also considered safe by the World Health Organization (WHO) for use in aircraft for vector control (Rayman, 2006). WHO also recommends certain pyrethroids (bifenthrin, cyfluthrin, deltamethrin, lambda-cyhalothrin) for indoor residual spraying against malaria vectors (Walker, 2000; Raghavendra et al., 2011). In addition, WHO recommends treating bed nets with pyrethroids, as a crucial role in vector-based malaria intervention strategies (Raghavendra et al., 2011; WHOPES, 2005). Pyrethroids are also commonly used in developing countries, such as China, for pest control and disease vector eradication (Chen et al., 2011). Because pyrethroids are considered safer alternatives to OP pesticides, they assumed many roles formerly held by OPs, particularly for pest control in urban environments (Amweg et al., 2006). Pyrethroids are now widely used by professional pest control applicators as termiticides, for landscape application, or as perimeter treatments to keep pests out of structures. In addition, they are the dominant insecticides among retail sales to consumers (Weston et al., 2009). According to EPA usage data, approximately 1 million kg of permethrin are applied annually to agricultural, residential, structural and public health sites (Feo et al., 2010). Of this, more than 630,000 kg

are annually applied in nonagricultural settings, with 41% of applications made by homeowners on residential areas (EPA, 2006; Moore et al., 2009).

2.4 Pyrethroid use patterns

Databases that provide information on use patterns of pyrethroids are not readily available to the public in the United States. One exception is the California Department of Pesticide Regulation's (CDPR) Pesticide Use Reporting (PUR) database. CDPR began full use reporting of all agricultural pesticide applications in 1990 (CDPR, 2000). The program requires monthly reporting of any agricultural pesticide use to the county agricultural commissioners, who transfer the information to PUR. Data obtained from the PUR database suggest that in 2008 (the most recent year for which all data are available), the amount of pyrethroids professionally applied in non-agricultural situations in California was greater than for agricultural areas. A few pyrethroids, such as bifenthrin, fenpropathrin, and lambda-cyhalothrin, are used more frequently in agricultural settings. Overall, however, larger amounts of pyrethroids are used in non-agricultural settings than agricultural settings.

Using the same data from the PUR database reveals that landscape maintenance, public health pest control, and structural pest control are the three applications where pyrethroids were used most often in 2008. Data describing the non-agricultural uses of pyrethroids can be further classified into either "indoor," "outdoor," or "structural" use categories. The term "indoor uses" relates to disease control for humans and pets in buildings and homes. Pest control related to landscaping needs is covered by "outdoor uses". The "structural uses" category refers to pyrethroids being used to prevent structural damage to structures such as housing or commercial development from pests such as carpenter ants and termites. The amount of pyrethroids reported in the PUR database for each of these three use categories was summed, and the results suggested that "structural" use was greater than "outdoor" use, and both structural and outdoor uses of pyrethroids were two orders of magnitude greater than "indoor" uses of pyrethroids.

2.5 Emerging concerns

Pesticide resistance is the adaptation of pest populations targeted by a pesticide resulting in decreased susceptibility to that chemical. Over the past decade, a resurgence of bed bugs (*Cimex lectularius*) has occurred in North America, Europe, and Australia, with infestations now being common occurrences (Romero et al., 2007). Romero et al. (2007) suggested that the difficulties associated with controlling bed bug infestations, and the survival of field-collected bed bugs after direct spray applications with label-rate formulated pyrethroids including deltamethrin and lambda-cyhalothrin, indicates that bed bug resistance to pyrethroid insecticides may be widespread. Malaria vector control is dependent on pyrethroid insecticides, as this is the only class of chemicals approved for use on insecticide-treated nettings. However, there has been a dramatic increase in reports of pyrethroid resistance in malaria vectors over the past decade; the resistance alleles are spreading at an exceptionally rapid rate throughout Africa (Ranson et al., 2011).

Synthetic pyrethroid insecticides have generated public concerns due to their increasing use and potential effects on aquatic ecosystems (Luo & Zhang, 2011). In recent studies, residues of pyrethroid insecticides have been detected in the sediment from a number of urban streams in California at levels toxic to *Hyalella azteca* (Weston et al., 2005; Amweg et al., 2006;

Oki et al., 2007; Budd et al., 2007; Weston & Lydy, 2010). Although pyrethroids offer lower toxicity to human applicators and nontarget mammals and birds than OPs, they are highly toxic to invertebrates and fish (Palmquist et al. 2010). This lead the CDPR, which regulates pesticide sales and use in California, to initiate a re-evaluation of more than 600 products containing pyrethroid pesticides in 2006 (CDPR, 2007).

The increasing and widening use of pyrethroid insecticides necessitates a thorough understanding of the environmental fate and ecological effects of their use. In many ways, these insecticides represent a significant improvement over other insecticide classes, exhibiting lower mammalian and avian toxicity and better selectivity to target species than the OPs and less persistence than the organochlorine insecticides. Additionally, the unique mode-of-action and chemical behavior of pyrethroids suggests that ecotoxicological effects and fate in the terrestrial and aquatic environment are likely to be significantly different from that of their OP precursors. Subsequently, a review of the current literature regarding environmental fate and bioavailability of the pyrethroid insecticides and their ecotoxicity, with special reference to sublethal and ecological effects is presented.

3. Environmental fate in the terrestrial environment

Pyrethroid releases into the terrestrial environment occur largely via spray drift from both agricultural and non-agricultural applications, although accidental spills and direct application to soil surfaces can also be considered sources of release. However, because of their strong tendency to adsorb to soils and organic matter, these compounds are unlikely to undergo significant migration from areas of direct application, except on particulates that are carried by wind or water.

Pyrethroid persistence under aerobic soil conditions (e.g., expected field conditions) is highly variable, with half lives ranging from 11.5 days for cyfluthrin to 96.3 days for bifenthrin (Oros & Werner, 2005). Photolysis is likely a significant degradation pathway for pyrethroids in the soil, and is influenced by soil characteristics. The half life of esfenvalerate in different soil systems was significantly increased under dark conditions, with half-lives of 7.8 to 100.0 days under continuous irradiation versus 150.0 to 553.4 days in the dark (Katagi, 1991). Analysis of soils indicated that esfenvalerate was largely present in complexes with humic acid. Further, the fraction of fenvalerate bound to soil particles was greater in silty clay loam soil as compared to sandy loam soil (Lee, 1985). Consequently, it is probable that photolytic degradation of pyrethroids could proceed more slowly in highly organic soils. Microbial degradation also appears to be a significant breakdown route. Degradation of pyrethroid insecticides was observed to be more rapid in natural versus sterilized soils, indicating that biological processes do contribute to breakdown in soil. Eight weeks after treatment, fenpropanate, cypermethrin, and permethrin concentrations were reduced to 20% of the original amount in natural mineral and organic soils, while sterilized systems retained more than 80% of initial concentrations (Chapman et al., 1981). Authors concluded that soil microorganisms were largely responsible for the more rapid rate of degradation in natural soils. There is some evidence that the byproducts of pyrethroid degradation are more mobile in soils than are the parent compounds (Kaufman 1981, Lee 1985). However, these compounds are likely to be significantly less toxic than the parent insecticide.

Soil concentrations of pyrethroids may be reduced as a result of interception by the plant canopy. For instance, the residues of cypermethrin in agricultural field soils under crop cover were determined to be approximately one-tenth of those in bare soil following a spray

event (Wiles & Frampton, 1996). Subsequently, a large fraction of spray drift may become associated with plant surfaces and undergo different degradation processes. The observed half life of cypermethrin on elm bark was approximately 50 days, and resulted in improved efficacy against bark beetles, reducing the need for additional applications. Wiles & Jepson (1994) also determined that pyrethroid residues are more persistent on leaves, and that plant-dwelling insects may therefore be exposed to pyrethroid residues longer than soil-dwelling invertebrates.

4. Effects on terrestrial invertebrates

Potential terrestrial invertebrate pyrethroid receptors can be grouped in two categories—pest invertebrates and nontarget beneficial species that include pollinators and predaceous insects. Applications to pest invertebrates are intentional and designed to result in maximum mortality of target organisms; exposures to nontarget organisms, however, are incidental and largely unintentional. In both cases, this review will focus on the sublethal effects observed following pyrethroid exposure, as sublethal exposures are much more common under environmentally-relevant exposure scenarios than are lethal concentrations.

4.1 Pest invertebrates

Pyrethroids provide excellent control of pest invertebrates as a result of residual activity, strong repellency, and high toxicity (Hammond, 1996). For example, when compared with OP insecticides, pyrethroids provide more effective control of the apple pest, the tufted bud moth (*Platynota idaeusalis)* (Hull et al., 1985). Hall (1979) reported that pyrethroids provided equivalent or superior control of plum curculio (*Conotrachelus nenuphar*), codling moth (*Cydia pomonella*) and redbanded leafroller (*Argyrotaenia velutinana*) when compared with OPs.

Sublethal pyrethroid exposure has been shown to interfere with both physiological growth processes and the ability to feed in insect pests. Sublethal doses of d-phenothrin (5 ng/day) were linked to significant reductions in lipid synthesis in the assassin bug (*Triatoma infestans*) (Juarez, 1995), indicating that processes critical to insect growth were disrupted by pyrethroid exposure. Similarly, exposure to sublethal doses of deltamethrin altered glucose metabolism in the migratory locust, with significant increases in the conversion of glucose to carbon dioxide, potentially as a result of metabolic hormone release following stimulation of neurohemal organs by pyrethroid exposure (Moreau et al., 1987). Food acquisition also is affected by consumption of pyrethroid residues. Dietary exposure to synthetic pyrethroids including cypermethrin, deltamethrin, fenvalerate and permethrin decreased feeding rates in the red flour beetle (*Tribolium castaneum*), a pest of stored grain, which resulted in reduced larval weight, delayed pupation and delayed adult emergence (Ishaaya et al., 1983). Together, these results suggest that non-lethal pyrethroid exposures can significantly alter normal invertebrate growth processes.

In addition to interfering with growth processes, pyrethroid exposure has also been linked to disruption of invertebrate chemical communication. Adult tobacco hornworm (*Manduca sexta*) females exposed to permethrin were less likely to attract males than were non-exposed females (Haynes, 1988). Permethrin also reduced male oriental fruit moth (*Grapholita molesta*) response to pheromones in a wind tunnel (Haynes, 1988). Similarly, permethrin was found to interfere with the chemical communication of male and female

pink bollworm moth (*Pectinophora gossypiella*) (Haynes and Baker, 1985). In both males and females, disruption of sex pheromone communication was a more sensitive endpoint than disruption of flight abilities.

There is also evidence that pyrethroid exposure can reduce the number and viability of eggs produced by pest invertebrates. Egg development of engorged female African ticks (*Amblyomma hebraeum*) was significantly altered by cypermethrin-exposure. Doses as low as 0.05 µg cypermethrin per tick reduced average ovary weight, average oocyte length, and ovary vitellogenin (Friesen and Kaufman, 2003). Likewise, deltamethrin also reduced the production of both ootheca and viable offspring by German cockroaches (*Blattella germanica*) (Lee et al., 1998). Longevity of exposed male and female roaches was significantly reduced by deltamethrin exposure, an effect that is likely to further reduce fecundity.

4.2 Beneficial invertebrate species

Application of pyrethroids to agricultural fields can result in the unintentional exposure of beneficial invertebrate species, such as pollinators, parasitoid wasps, generalist predators, and soil decomposers, as a result of their proximity to sprayed areas. Although such species may be exposed to acutely lethal concentrations of insecticides, it is far more probable that the majority of nontarget invertebrates will encounter sublethal concentrations. These levels of exposure can impact foraging and prey-location behaviors, development, growth, and reproduction.

4.2.1 Honeybees

As domesticated pollinators, honeybees (*Apis mellifera*) are common residents of agricultural areas. Despite measures to prevent their direct exposure to pesticides (e.g., forgoing pesticide application during peak blooming and bee foraging times), this proximity to sprayed areas increases the probability of both cuticular and dietary exposure of honeybees to insecticides, including pyrethroids. Pyrethroid insecticides, in particular, have been demonstrated to interfere with important honeybee behaviors and with reproductive output.

Low exposures of permethrin alter honeybee behavior associated with maintenance, feeding, and communication. Bees receiving topical applications of 0.001 µg permethrin were more likely than non-exposed bees to engage in self-cleaning, leg-rubbing, trembling dances, abdomen tucking and rotation (Cox & Wilson, 1984). Permethrin-intoxicated bees also spent less time walking, giving food, and antennae touching. Similarly, forager honeybees exposed to 2.5 ng deltamethrin/ bee (27 times less than the LC_{50}) exhibited altered flight patterns and homing abilities as compared to non-exposed bees (Vandame et al., 1995). While approximately 90% of control bees returned to the hive within 30 seconds of flight, only 9.0% of treated bees were able to return within this time period. Authors also noted that this delay was most likely related to bees' inability to correctly orient themselves using the sun after being exposed to deltamethrin.

Dietary exposures to sublethal pyrethroid concentrations (i.e., as in nectar or syrup) also cause behavioral aberrations, and decrease fecundity. Feeding on syrup containing 940 µg/L deltamethrin reduced the proportion of bees exhibiting learned orientation towards an odor stimulus by approximately 11% to 24% (Decourtye et al., 2005). Consumption of diet containing bifenthrin or deltamethrin at concentrations of 4.0, 7.9, 15.5, 30.6, and 60.2 mg/L or 20.0, 36.0, 64.8, 116.6, and 210.0 mg/L, respectively, also caused sublethal effects in

honeybees. Daily egg production was reduced by consumption of both bifenthrin and deltamethrin, and time in the egg stage was extended. Deltamethrin exposure also reduced capping rate and extended the duration of the immature stage (Dai et al., 2010). Other pyrethroid effects occurred sporadically, as effects on rate of development, number of feeding larvae (unsealed brood stage), hatch rate, and number of post-feeding larvae and pupae (sealed brood stage), were not significant in every year tested.

4.2.2 Parasitoids

Like honeybees, parasitoid wasps are beneficial hymenopterans that frequent agricultural fields or other commonly sprayed areas. These insects use many common agricultural pests as "nests" for their developing offspring, laying their eggs inside aphids, larval moths, and beetles. After hatching, the parasitoid larvae consume the host from the inside, eventually causing death. The species-specific nature of the parasitoid-host relationship increases their economic benefit: whereas generalist predators may consume non-pest as well as pest prey items, parasitoids are host-specific, thereby providing better control of targeted pest species. However, exposure to sublethal concentrations of pyrethroids has been observed to interfere with parasitoid host-finding behaviors, reaction to mating pheromones, and reproduction.

Olfactory orientation toward prey populations was reduced in aphid parasitoids following exposure to sublethal concentrations of lambda-cyhalothrin (Desneux et al., 2007). Similarly, the addition of deltamethrin to honeydew residues also caused significant parasitoid repellency (Longley & Jepson, 1996). In addition to altering the ability of parasitoids to locate their hosts, deltamethrin exposure at 10% of the LD_{50} also altered sex pheromone production by the female moth egg parasitoid *Trichogramma brassicae*; males localized less precisely to pheromone extracts from exposed females than from unexposed females (Delpuech et al., 1999).

Although an inability to locate hosts or mates can cause a reduction in parasitoid fecundity, there is evidence that pyrethroid exposure can also alter other aspects of parasitoid reproduction. When exposed to lambda-cyhalothrin prior to oogenesis, the Trichogramma wasp (*T. pretiosum*) experienced a reduction in fecundity (Desneux et al., 2007). Interestingly, sublethal deltamethrin and lambda-cyhalothrin exposures resulted in decreased number of female offspring and altered male:female ratios in exposed wheat aphid parasitoids (*Aphidius uzbekistanicus*). Authors noted that in this hymenopteran species, females are produced by fertilized eggs, while unfertilized eggs produce males, and that the behaviors associated with egg fertilization may have been impacted by the insecticide exposure (Desneux et al., 2007). However, data from field studies indicate that sublethal effects of pyrethroid exposure may not have large-scale impacts on overall parasitoid population viability (Li et al., 1992, Longley, 1999).

4.2.3 Other beneficial invertebrate species

Similar effects on insect behavior and feeding have been demonstrated in generalist predator species. Increased grooming has been observed in ladybird beetles (*Coccinella septempunctata*) following sublethal pyrethroid exposure. When released in areas previously sprayed with deltamethrin, these insects were observed to groom and walk more frequently (Desneux et al., 2007). Further, the prey attack rate of the 'sit-and-wait' assassin bug (*Acanthaspis pedestris*) was reduced by a factor of 2.4 to 6.4 following exposure to cypermethrin at suggested field application rates (Desneux et al., 2007). Pyrethroid exposure of prey items can also cause anti-feeding responses in invertebrate predators. Predatory

carabid beetles (*Nebria brevicollis*) were more likely to regurgitate deltamethrin-exposed aphids as compared to untreated prey. Exposure to sublethal concentrations of permethrin decreased the developmental time of female stink bugs (*Supputius cincticeps*), but extended that of the males (Desneux et al., 2007).

However, field cypermethrin soil residues were found to cause less than 10% mortality to Collembola in laboratory studies, and this insecticide was less toxic than chlorpyrifos, an OP insecticide, to Collembola (Wiles & Frampton, 1996). Toxicities of leaf and soil deltamethrin residues to beneficial insect species, including predatory beetles and parasitoids, were determined to be different, with leaf residues exhibiting higher toxicity. The LD_{50} values for ground beetles (*Demetrias atricapillus)*, rove beetles (*Tachyporus hypnorum)*, ladybird beetles (larvae and adults), hoverflies (*Episyrphus balteatus)*, and brachonid wasps (*Aphidius rhopalosiphi)* exposed to deltamethrin residues on leaf surfaces were >50, 1.2, 0.4, 2.0, 4.8 and 7.1 g a.i./ha, respectively, (Wiles & Jepson, 1994). Tested species appeared less susceptible to soil residues. These results indicate that plant-active predators are likely to be more sensitive to pyrethroid applications than are soil-dwelling predatory insects.

5. Effects on terrestrial vertebrates

One of the primary benefits of the use of pyrethroids is the compounds' low inherent toxicity to a number of nontarget terrestrial vertebrates, including mammals and birds. Given this, a discussion of avian and mammalian pyrethroid toxicity is unnecessary in the context of environmental exposures, as use rates are low enough to preclude any effects on these receptors. However, less is known regarding the toxicity of pyrethroids to terrestrial reptilian species.

Brown tree snakes (*Boiga irregularis*) were sensitive to the effects of commercially available pyrethroid insecticide formulations applied dermally as a 2-second spray. Within one hour of exposure, intoxicated snakes exhibited muscle tremors, disorientation, and eventual paralysis, followed by death (Brooks et al., 1998). Unsurprisingly, the pyrethroid synergist piperonyl butoxide was observed to enhance the toxicity of pyrethroid formulation to snakes, and a 2-second spray application delivered a high amount of active ingredient (102 to 320 mg/kg bw). Sensitivity to pyrethins was determined to be temperature-dependent in green anole lizards (*Anolis carolinensis*), a phenomenon also observed in exposed invertebrates. Lizard mortality following a 2-second bath in 300 mg /L pyrethrin solution was 30% at 38°, but increased to 100% at temperatures below 20°C (Talent, 2005). These results may indicate an increased reptilian susceptibility of pyrethroids in cooler temperate climates.

6. Effects on terrestrial communities

Little research has been conducted concerning the effects of pyrethroids on nontarget terrestrial communities. Because bird and mammals species are largely unaffected by pyrethroids, it may be that the impacts upon terrestrial ecosystems are limited to invertebrate communities. In fact, Ali et al. (2011) report that adverse effects of pyrethroid use on bird populations almost always occurs through a destruction of the food supply of insectivorous birds. Hence, effects in the vertebrate community will most likely only be observed following extremely heavy and widespread uses of these compounds that would result in the near-obliteration of resident invertebrates. Further, while the effects of

pyrethroids on nontarget invertebrate fitness have been extensively investigated, most studies focus on the effects to specific beneficial species; fewer studies have been conducted concerning the effects upon field invertebrate communities.

Although effects on nontarget soil arthropod communities are likely to be minimal and transient, pyrethroid impacts on plant-dwelling insect communities are more significant. Cypermethrin applied at levels sufficient to control aphids (25 g a.i./ha) also suppressed numbers of predaceous carabid beetle populations for 6 days post-spray. However, the pyrethroid had no effects on staphliniid beetles and only minor effects on predatory flies and parasitic wasps (Inglesfield, 1989). Applications of deltamethrin at 6.23 g a.i./ha similarly reduced ladybird beetle populations in wheat crops, but had no impact on fly or parasitic wasp species. Communities in areas adjacent to sprayed fields may also come into contact with pyrethroids in the form of spray drift. The survival of the aphid parasitoid *Aphidius colemani* in edge-of-field areas was impacted by spray drift from two applications of the lamba-cyhalothrin formulation Trafo® at the recommended rate of 10 g a.i./ha (Langhof et al., 2003). Proportions of hoverfly (Syrphidae) eggs to aphids and percentages of aphid mummies increased with distance from edge-of-field, although these trends were not significant. Authors concluded this was likely a result of the non-homogenous distribution of lamba-cyhalothrin spray drift residues (Langhof et al., 2003). Subsequently, effects observed in edge-of-field areas probably will not be consistent between different fields, as the variables affecting the magnitude and distance of insecticide drift are highly site-specific.

7. Environmental fate and bioavailability in aquatic systems

Pyrethroids are most commonly introduced into aquatic systems via runoff from sprayed fields, lawns, parking lots, etc., during rainstorm events, and, to a lesser extent though spray drift. Regression modeling of pesticide loading in the Sacramento River indicated that the frequency and magnitude of pyrethroid use as well as precipitation patterns are critical factors that govern pyrethroid transport to surface waters (Oros & Werner, 2005). However, field-specific variables determining breakdown rates (e.g. canopy cover, temperature, persistence on soil surface) in relation to precipitation events also play a role in determining pyrethroid concentration in runoff. It has also been proposed that transportation through concrete drainage systems that are present in suburban and urban areas, may result in the transport of greater concentrations of aqueous-phase pyrethroids, as compared with particulate-rich agricultural runoff channeled through earthen ditches (Weston & Lydy, 2010).

7.1 Fate of pyrethroids in aquatic systems

Pyrethroid insecticides are strongly hydrophobic; the log K_{ow} values for 11 different pyrethroid compounds ranged from 4 for esfenvalerate to 7.6 for tralomethrin (Oros & Werner, 2005). As such, the water-soluble fraction of pyrethroids introduced into an aquatic system will be short-lived and quickly reduced. A mesocosm study of the effects of lambda-cyhalothrin spray drift indicated that the pyrethroid insecticide rapidly dissipated from the water column (half life of approximately one day), leading authors to theorize that aqueous pyrethroid exposure would cause only short-term community stress (Arts et al., 2006). Subsequently, much of the fate and transport of pyrethroids in aquatic systems is governed by particulate adsorption.

Pyrethroid transport within aquatic systems occurs through movement of pyrethroid-absorbed fine particulates (Gan et al., 2005). Although the half-lives of most pyrethroid insecticides are in the order of days to weeks in the water column, pyrethroids adsorbed to particulates are considerably more persistent, with reported half-lives on sediments of 150 to 200 days (Amweg et al., 2005). Pyrethroids in stream water were most frequently associated with suspended solids and particulates, with only 0.4% to 1.0% of added pyrethroids present in the freely dissolved phrase. In runoff, the freely dissolved phase accounted for 10% to 27% of the total pyrethroid mass (Liu et al., 2004). Following pyrethroid addition to stream water, greater than 97% of the total mass added was sorbed to suspended solids and particulates.

Ditches receiving agricultural runoff mitigated bifenthrin and lambda-cyhalothrin contamination by promoting their retention and adsorption. Although runoff initially contained 666 µg/L bifenthrin and 374 µg/L lambda-cyhalothrin, this was reduced to 7.24 and 5.23 µg/L, respectively, 200 m downstream. At 400 m, no pyrethroid residues were detected (Bennett et al., 2005). In general, pyrethroid concentrations in sediment increased with distance along a drainage channel downstream from a sedimentation pond. While bifenthrin sediment concentrations in the pond averaged 0.33 mg/kg, these increased to 2.27 mg/kg 104 m downstream and 10.64 mg/kg at 145 m downstream. A similar pattern was observed for the distribution of *cis*-permethrin, with sedimentation pond concentrations of 0.77 mg/kg increasing to 1.1 and 4.45 mg/kg at 104 and 145 m downstream, respectively (Gan et al., 2005). Enrichment of pyrethroid concentrations downstream from a sedimentation pond correlated to increasing organic carbon and clay content of the sediment. As pyrethroids tightly adhere to fine organic particulates, it is probable that pyrethroid-bound fines were more likely to be transported by downstream flow, resulting in downstream enrichment.

Concentrations of cypermethrin, deltamethrin, fenvalerate, and permethrin in the interstitial (pore) water of sandy sediment were five to six times as high as concentrations measured in the overlying water. However, the differences between pyrethroid concentrations in overlying and pore water in silt or clay sediments were considerably lower, a 1.3 to 1.5-fold difference (Muir et al., 1985). Consequently, benthic and epibenthic invertebrates in systems with clay or silt sediments are likely to experience similar pyrethroid exposures. However, exposure of benthic organisms in sandy substrates may not be greater despite the higher total concentration of pyrethroids a result of chemical adsorption to the larger particles; the mass adhered to sand is likely not as bioavailable to sediment-dwelling invertebrates (You et al., 2008).

7.2 Bioavailability of pyrethroids in aquatic systems

Although pyrethroids exhibit strong hydrophobicity and therefore would be expected to bioaccumulate in aquatic organisms (and even to biomagnify in the food chain), they are rapidly depurated, and consequently do not bioaccumulate (Oros & Werner, 2005). Moreover, the hydrophobic nature of these chemicals results in binding to dissolved organic carbon (DOC), which removes them from the aqueous phase of the water column and causes them to bind to many sediments, thus reducing their bioavailability in aquatic systems.

Addition of DOC to aqueous laboratory systems reduced the bioaccumulation and toxicity of deltamethrin, fenvalerate, and cyhalothrin in the cladoceran *Daphnia magna*. Low DOC

concentrations (1.76 mg/L) sequestered up to 76.4% of added fenvalerate and 80.8% of added deltamethrin after 24 hours (Day 1991). Similarly, exposure to lambda-cyhalothrin in a water-sediment system reduced toxicity to *Daphnia magna* and carp (*Cyprinus carpio*) by factors of 175 and 74, respectively, over that observed in water-only exposure scenarios (Maund et al., 1998). This indicates that laboratory studies are likely to overestimate the toxicity of pyrethroids to field populations. Following introduction into a water-sediment system, nearly 99% of cypermethrin was adsorbed to sediment within the first 24 hours, and binding to sediments reduced the bioavailability of the insecticide to *Daphnia magna* and *Chironomus tentans* (Muir et al., 1985).

The bioavailability of pyrethroids to sediment-dwelling species is commonly understood to be largely influenced by the sediment organic carbon (OC) content. Consequently, sediment pyrethroid concentrations are frequently OC-normalized. However, bioavailability of pyrethroids to benthic invertebrates also is influenced by the presence of leaf and plant material. Toxicity of bifenthrin to *Hyalella azteca* was reduced by the addition of leaf material to sediments, even when normalized to OC content. Authors noted that this amphipod preferentially inhabits leaf material, and that this association may reduce exposure to the pyrethroid (Maul et al., 2008b).

In addition to the quantity of OC in sediments, the type of the OC (e.g., in terms of the number of binding sites and general affinity for pyrethroids) is an important determinant of bioavailability. Subsequently, differently sourced sediments can exhibit different adsorptive capacities for pyrethroids despite having the same OC content. Amweg et al. (2005) collected sediments from several different field sites in the Central Valley of California to determine the pyrethroid-mediated toxicity to *Hyalella azteca* in a series of laboratory bioassays. Despite normalizing the pyrethroid LOAEC and LC_{50} values to organic carbon content, the toxicity threshold was different for different sources (Amweg et al., 2005). Similarly, the toxicity of permethrin to chironomid larvae in artificial sediments was greater than in naturally-derived sediments. In natural systems containing 200 ng/g permethrin, chironomid emergence was reduced to approximately 80% of controls, while in artificial peat and α-cellulose sediment, midge emergence at the same pyrethroid concentration was approximately 62% and 8%, respectively (Fleming et al., 1998). In addition, toxicity of deltamethrin to *Chironomus riparius* larvae in spiked sediment systems was reduced by use of natural sediments versus artificial sediment. This difference was attributed to the higher organic content in the natural sediment (12.5%, as opposed to 4.8% in artificial sediment). Additionally, during the 10-day bioassay, 50% of the added deltamethrin was degraded in the natural sediment system, while no degradation was observed in the artificial sediment. However, analysis of sediment pyrethroid concentrations indicated that lower toxicity in natural sediments did not result from rapid degradation, but from reduced bioavailability (Åkerblom et al., 2008). Survival of *Chironomus riparius* larvae in permethrin-spiked sediments was greater in peat sediments than in α-cellulose sediments, likely due to different binding capacities and affinities for the pyrethroid between the peat and α-cellulose (Fleming et al., 1998). Subsequently, pyrethroid bioavailability may be highly site-specific and dependant on OC source as well as content.

8. Aquatic ecotoxicity of pyrethroids

Although reasonably non-toxic to avian and mammalian receptors, pyrethroids exhibit very high toxicity to nontarget invertebrates and fish, making them vulnerable to low

concentrations of pyrethroids entering surface waters via runoff or spray drift. In addition, aquatic species may be chronically exposed to sediment-associated pyrethroid insecticides, as sediments function as a sink for these compounds, although this depends upon sediment type and organic matter, as discussed above. Consequently, the aquatic toxicity of this class of insecticides is of significant concern.

8.1 Effects of pyrethroids on nontarget aquatic invertebrates

8.1.1 Impacts on aquatic invertebrate behavior

Given that the pyrethroid mode of action is on the peripheral nervous system, it is not surprising that behavioral effects in exposed invertebrates are numerous and distinctive, often occurring within minutes of exposure. This disruption of behavioral homeostasis can alter feeding rates, cause loss of coordination and paralysis, or stimulate hyperactivity that may increase exposure to predators, both of which can have ecologically-relevant consequences.

Surface insects were observed to be susceptible to pyrethroid over-spray, exhibiting a rapid loss of coordination and paralysis in response to esfenvalerate only a few hours after application (Samsøe-Petersen et al., 2001). Following sublethal aquatic exposures to cypermethrin, cyfluthrin, and deltamethrin, red swamp crayfish exhibited similar responses (Morolli et al., 2006). Likewise, sublethal permethrin exposure (0.03 to 0.05 μg/L) altered behavior in exposed caddisflies (*Brachycentrus americanus*) and stoneflies (*Pteronarcys dorsata*). Caddisflies exhibited increased "pawing" motions followed by a cessation of feeding behaviors, while intoxicated stoneflies lost equilibrium and became immobilized (Anderson, 1982). In both cases, mortality occurred several days after exposure, and the author theorized that disrupted feeding and starvation may have caused mortality.

Pyrethroid exposure has also been demonstrated to interfere with the complex building behaviors exhibited by case-building caddisflies (*Brachycentrus americanus*); these cases provide protection from predation, improve respiration and act as a refuge during pupation. Following 48-hour esfenvalerate exposures of 0.05 μg/L and greater, caddisfly larvae were observed to exit their cases, an abnormal behavior not observed in non-exposed larvae (Johnson et al., 2008). More significantly, case-rebuilding behaviors were also negatively affected by esfenvalerate exposure.

8.1.2 Impacts on aquatic invertebrate growth

Sublethal exposures to toxicants can lead to a reduction in growth. Impaired organisms may experience a reduction in feeding, either through chemical deterrence or via disrupted feeding behaviors. Conversely, exposure to toxicants may require the expenditure of additional energy for detoxification, metabolism, and restoration of behavioral and physiological homeostasis. Alteration of organisms' energy budgets following pyrethroid exposure can be observed as changes in growth and dry mass.

Short-term (24-hour) fenvalerate exposures as low as 0.3 μg/L reduced *Daphnia magna* food filtering rates (Reynaldi et al., 2006). Although individuals were eventually able to recover from the effects of the pulsed pyrethroid exposure and resume normal feeding behavior, exposed individuals were smaller and required more time to mature than unexposed organisms. Sediment-bound pyrethroids also adversely affected the growth of *Hyalella azteca* amphipods in laboratory experiments. Amphipod growth LOAECs ranged from 0.08 to 0.21 μg/g OC for bifenthrin, 0.46 to 0.77 μg/g OC for cyfluthrin, 0.20 to greater than 1.57 μg/g

OC for deltamethrin, 0.29 to 0.49 µg/g OC for esfenvalerate, 0.14 to 0.23 µg/g OC for lambda-cyhalothrin, and 0.68 to 5.3 for permethrin (Amweg et al., 2005). Although not tested, these reductions in growth are likely to affect later size and fecundity. Maul et al. (2008a) noted that pyrethroid-mediated reductions in growth had later effects on Chironomid fitness. Both larval ash free dry mass (AFDM) and instantaneous growth rate (IGR) were negatively impacted by exposure to sediment contaminated with bifenthrin, lambda-cyhalothrin, or permethrin, with respective 10-day LOAECs of 2.2, 2.0 and 74.2 µg/g OC. Reduced growth at these exposures was linked to reduced emergence, adult size, and fecundity (Maul et al., 2008a).

Exposure to pyrethroids has been demonstrated to impact specific invertebrate growth processes. The timing of mysid and pink shrimp mortality during acute toxicity tests with cypermethrin, permethrin, and fenvalerate appeared to be linked to molting, suggesting an increase in sensitivity to pyrethroid during this growth process (Cripe, 1994). Likewise, molting failures were noted in *Daphnia magna* exposed to cypermethrin, and this appeared to contribute to the mortality rates associated with the insecticide exposure (Kim et al., 2008). It is probable that young or rapidly growing arthropods may be more susceptible to the toxic effects of these insecticides, because they are likely to undergo more frequent molts than slow-growing and larger invertebrates. Similarly, those invertebrates that undergo specialized growth and re-generation may also be especially susceptible to the effects of sublethal pyrethroid exposure. For instance, chronic exposures to permethrin (<2 µg/L) disrupted male fiddler crab (*Uca pugnax*) limb re-generation, although female limb development was unaffected (Stueckle et al., 2009).

8.1.3 Effects on aquatic insect emergence

While delayed toxicity is rarely measured during the course of standard acute aquatic toxicity testing, this endpoint is critical in understanding the total effect of toxicants on aquatic organisms. For semi-aquatic insects, delayed or suppressed emergence has been observed following sublethal exposures to pyrethroids. This effect can alter normal male:female ratios and reduce reproductive output, resulting in delayed consequences of exposure.

Timing of caddisfly (*Limnephilus lunatus*) emergence was altered following larval exposures to 0.001 µg/L fenvalerate for one hour, although a reduction in emergence rate was only observed after exposure to a one-hour pulse of 0.1 µg /L fenvalerate (Schulz & Liess, 2000). Adult dry weight was reduced by larval exposure to 0.01 µg /L fenvalerate for one hour. Although all of these delayed effects have the potential to alter reproductive output and population dynamics, these data suggest that emergence timing is the most sensitive endpoint. Likewise, pulsed esfenvalerate exposure also had significant long-term impacts on the mayfly *Cloeon dipterum*. Reduced adult emergence of mayflies was observed 29 days after one-hour exposures to 0.01 µg/L esfenvalerate, a concentration three orders of magnitude lower than concentrations determined to result in acute mortality in this species (Beketov & Liess, 2005). Interestingly, the emergence of *Chironomus riparius* larvae from artificial ponds was stimulated at low esfenvalerate concentrations (0.1 and 0.2 µg/L), but was delayed by concentrations of 0.8 µg/L and greater (Samsøe-Petersen et al., 2001). However, given that the life cycles of semi-aquatic insects are finely attuned to seasonal and environmental cues, improperly timed emergence can result in new adults emerging into inhospitable climatic conditions.

There is some evidence that a reduced emergence rate following pyrethroid exposure may be related to physical or physiological difficulties in exoskeleton shedding. Reductions in mayfly (*Cinygmula reticulata*) emergence following 48-hour esfenvalerate exposures of 0.005 to 0.015 µg/L were a result of increases in unsuccessful molting (Palmquist et al., 2008b). Affected insects appeared unable to shed nymphal exoskeletons, which led to death; a similar response was observed for *B. americanus* caddisfly emergence after 48-hour pupal exposures of 0.1 µg/L esfenvalerate and greater (Palmquist et al., 2008b).

8.1.4 Effects on aquatic invertebrate reproductive success and output

Exposure to sublethal concentrations of pyrethroids affects the reproductive output of a number of aquatic invertebrate species, through simple reductions in the number of eggs produced, disruption of complex mating behaviors, or alteration of male to female ratios.

Short-term esfenvalerate exposures reduced the fecundity of both mayfly and caddisfly species. One-hour exposures to esfenvalerate (0.01 to 0.1 µg/L) followed by low food availability reduced egg production in female *Cloeon dipterum* mayflies (Beketov & Liess, 2005). Similarly, 48-hour pupal exposures to 0.05 µg/L esfenvalerate and greater reduced the ratio of egg weight to total female body weight in *B. americanus* caddisflies (Palmquist et al., 2008b). Chronic exposure to fenvalerate concentrations of 0.005 µg/L increased *Daphnia galeata mendotae* longevity by an average of 14 days, but reduced brood size by approximately 5 individuals. Exposures of 0.01 µg /L fenvalerate and greater over the daphnid life cycle reduced longevity, total reproductive output, brood size, and brood number (Day, 1989). Continuous (21-day) exposure to 0.3 µg/L fenvalerate was also demonstrated to have a greater adverse impact on *D. magna* reproductive rate than pulsed exposures to higher concentrations, and prevented the recovery of the exposed population (Reynaldi & Liess, 2005).

Sublethal pulsed exposure to esfenvalerate negatively affected both the fecundity and mating behavior of *Gammarus pulex* amphipods. Although 100% of test organisms survived one-hour pulses of up to 2 µg/L esfenvalerate, pulsed concentrations as low as 0.01 µg/L reduced the number of intact mating pairs (Cold & Forbes, 2004). Exposure to short-term pulses of 0.05 µg /L esfenvalerate and greater also increased mating pair reformation time by a factor of six. Delayed mating pairs also produced fewer offspring than non-intoxicated amphipods (Cold & Forbes, 2004).

8.1.5 Effects of dietary exposure to pyrethroids

Given the hydrophobicity of pyrethroid insecticides, the primary route of field exposure for water column invertebrates may be consumption of insecticide-contaminated diet. Despite this, few studies have been conducted on the dietary toxicity of this class of compounds. Undoubtedly, some fraction of toxicity observed in invertebrate sediment exposure studies does result from consumption of pyrethroid-contaminated material, but separation of dietary uptake from cuticular uptake under these scenarios is difficult.

Palmquist et al. (2008a) examined the impact of consumption of esfenvalerate-exposed dietary items on 3 species of aquatic insects. No evidence of feeding deterrence was observed, as none of the species tested rejected the esfenvalerate contaminated food. After three weeks, mayfly nymphs (*Cinygmula reticulata*) fed on algae pre-exposed for 24-hours to 0.5 to 1.0 µg/L esfenvalerate were smaller than control organisms. Further, consumption of diet exposed to 1.0 µg/L esfenvalerate for 10 days by final instar *C. reticulata* nymphs reduced the number and length of eggs in emerged females (Palmquist et al., 2008a).

Caddisfly (*B. americanus*) larvae feeding on dead esfenvalerate-contaminated *C. tentans* larvae exhibited characteristics of pyrethroid toxicity, including case abandonment. Pre-exposure of dietary items to 0.75 and 1.0 µg/L esfenvalerate for 24 hours resulted in increased rates of case-abandonment and mortality following consumption (Palmquist et al., 2008a). Although the dietary pyrethroids concentrations necessary to affect aquatic invertebrates appear to be significantly higher than aqueous concentrations, these results suggest that pyrethroids may still be toxicologically active when adsorbed to organic material.

8.2 Effects on aquatic vertebrates

Although designed to control pest invertebrate populations, pyrethroid insecticides are also toxic to nontarget aquatic vertebrates such as fish and amphibians (Ali 2011, Fishel 2005). Fish are considered especially sensitive to pyrethroid insecticides; similar behavioral and developmental effects have been observed in amphibians.

8.2.1 Amphibians

Documented symptoms in amphibians exposed to sublethal concentrations of pyrethroids include incoordination, lack of limb control, hyperactivity, tremors, and writhing. Leopard frog tadpoles exhibited convulsions and twitching following sublethal (1.3 µg/L) esfenvalerate exposure, with behavioral homeostasis re-established only one week after removal to clean systems (Materna et al., 1995). These behavioral aberrations, though not lethal alone, may increase predation of affected amphibians and fish.

Pyrethroid exposure also affects early amphibian development. Hatching success of moor frog (*Rana arvalis*) eggs was reduced following exposure to concentrations of α-cypermethrin as low as 1.0 µg/L, and a number of the exposed embryos exhibited convulsions and tail kinking following hatch. Cypermethrin concentrations as low as 0.1 µg/L during the egg stage also reduced length at metamorphosis (Greulich & Pflugmacher, 2003). Most notably, the jelly mass surrounding the amphibian eggs did not prevent embryos from absorbing cypermethrin from the surrounding aqueous medium. For instance, although exposure to either permethrin or fenvalerate at 0.01 to 2 ppm for 22 to 96 hours caused no increase in mortality, green frog (*Rana clamitans*) embryonic development was stunted at pyrethroid concentrations greater than 1 ppm, and operculum growth was also adversely affected (Berrill et al., 1993). Exposure to pyrethroids also causes reductions in tadpole biomass production 24 days following esfenvalerate exposures of 3.6 to 10 µg/L (Materna et al., 1995).

When exposed in a mesocosm study, amphibian fitness may actually improve as a result of decreased competition from invertebrate species. Green frog larval development was altered by a single pulsed exposure to 9 µg/L permethrin, and the tadpole Gosner developmental stage was increased, as was body mass (Boone, 2008). These responses indicated an increase in population fitness, and larger size and enhanced development would lead to earlier metamorphosis and larger metamorphic size. The author surmised that permethrin-mediated reductions in invertebrate populations led to increased algal food resources for developing amphibians (Boone, 2008). However, increases in amphibian biomass were also observed during single-species exposures. Permethrin concentrations of 1 µg/L increased the weight and size at metamorphosis of the common frog (*Rana temporaria*) tadpoles,

although exposure to esfenvalerate had no similar positive impacts on frog growth (Johansson et al., 2006).

8.2.2 Effects on fish

Impacts on normal behavior. As with aquatic invertebrates and amphibians, a number of significant behavioral effects have been observed in fish exposed to pyrethroids.

Low levels of pyrethroids caused neurobehavioral effects in young zebrafish (*Danio rerio*). Body spasms and uncontrolled swimming were observed following 6 day post fertilization exposures to either 1 µg/L deltamethrin, 1 µg/L cypermethrin, 10 µg/L cyhalothrin, 50 µg/L permethrin, or 100 µg/L bifenthrin (DeMicco et al., 2010) and Japanese medaka exhibited spinal curvatures and abnormal body shapes when exposed to cypermethrin concentrations as low as 100 µg/L (Kim et al., 2008). Although 4-hour esfenvalerate exposures as high as 20 µg/L caused no immediate mortality, abnormal behavior was observed in larval fathead minnows (*Pimephales promelas*) exposed to 0.455 and 1.142 µg/L esfenvalerate, including impaired swimming and feeding behaviors even after removal of exposed fish to clean systems (Floyd et al., 2008). Similarly, permethrin exposure was determined to induce a distinct set of behavioral effects in Japanese medaka (*Oryzias latipes*) juveniles, such as hyperactive swimming and midbody bends, followed by hypoactivity, reduced reaction to stimuli, and eventually death (Rice et al., 1997).

Chronic esfenvalerate exposure (0.01 to 0.05 µg/L) reduced the number of aggressive interactions between bluegill (*Lepomis macrochirus*) after 30 and 90 days of exposure; reductions persisted for at least 21 days following exposure (Little et al., 1993). Authors surmised that an increase in tremors interfered with normal inter-species interactions. Pulsed exposure to esfenvalerate also resulted in reduced aggression with increasing number of pulses (Little et al., 1993). However, bluegill sunfish inhabiting mesocosm systems dosed with a maximum 1.07 µg/L cyfluthrin (as the commercial product Baythroid®) exhibited no indications of behavioral sublethal responses to the exposure, as exposed fish were observed to swim, feed, and flee predation similarly to control fish (Morris et al. 1994).

Effects on fish reproduction and development. Sublethal pyrethroid exposures altered a number of reproductive and early developmental processes in fish. Low-level cypermethrin concentrations interfered with olfactory cues critical to Atlantic salmon reproduction. Five-day exposures of less than 0.004 µg/L inhibited the response of male fish to prostaglandin-type pheromones produced in the urine of reproductively-receptive female fish (Moore & Waring, 2001). In addition, when salmon milt and eggs were exposed to cypermethrin concentrations as low as 0.1 µg/L, the fertilization rate was reduced. Similarly, two pulsed exposures of 1 µg/L esfenvalerate delayed bluegill sunfish spawning (Little et al., 1993).

Gonadal development and structure in the freshwater snakehead fish, *Channa punctatus*, was altered by exposure to Devicyprin®, a commercial insecticide formulation containing 25% cypermethrin. Exposure equivalent to 1/10 of the LC_{50} for 10 days caused inflammation and intertubular vacuolization of testis, while necrosis of testis tissues was evident after 30 days of exposure (Srivastava et al., 2008). Ovary tissues exhibited vacuolization, stromal hemorrhage, and inflammation after 30 days exposure to 1/10 of the LC_{50}. In addition to effects on reproductive viability, pyrethroid insecticides also have been shown to alter embryonic and early life stage development in fish. Sublethal bifenthrin exposure caused increased spontaneous movement of zebrafish embryos that was linked to accelerated

hatching (Jin et al., 2009). In addition, 96-hour exposure to 109 µg/L or 256 µg/L bifenthrin increased incidences of curved body axis and pericardial edema. Later stages of Japanese medaka embryos were more sensitive to cypermethrin exposure, as most of the effects of the insecticide were observed at hatching (González-Doncel et al., 2003).

8.3 Community and ecosystem-level effects of aquatic pyrethroid exposures

Data garnered from higher-tier field and mesocosm studies provide an assessment of the true impacts of pyrethroid exposure in aquatic systems. Such experiments incorporate realistic measures of toxicant sequestration and degradation and also encompass interspecific competition and density-dependent compensatory (or depensatory) responses that can alter both toxic effects and long-term population dynamics.

8.3.1 Species sensitivity distributions

The risk of a specific toxicant to an aquatic community can be estimated through the construction of a species-sensitivity distribution (SSD) (Posthuma et al., 2002). SSDs combine toxicity values for a number of different species into a cumulative probability distribution that can be used to derive a community hazard level. These values ("hazard concentrations") are used to estimate the environmental concentrations that would be protective of 90 to 95% of the resident species (e.g., HC_5 or HC_{10} values).

Maund et al. (1998) compared the acute toxicities of lambda-cyhalothrin to a range of fish and arthropod species, including standard laboratory test species. For the fish species tested, the resulting HC_{10} was determined to be 0.087 µg/L, and the most sensitive species tested was the golden orfe (*Leuciscus idus*, 96-hour LC_{50} of 0.078 µg/L). This was in accordance with the lambda-cyhalothin HC_5 value reported in Maltby et al. (2005) for both fish and arthropod species, 0.003 µg/L; the maximum HC_5 value for pyrethroids was reported as 0.21 µg/L for permethrin. Interestingly, the range of fish sensitivities to lambda-cyhalothrin was much narrower than that for arthropods, as amphipods and isopods were roughly 100 times as susceptible to the pyrethroid insecticide as *D. magna* (Maund et al., 1998). However, the HC_{10} for the tested arthropod community was determined to be much lower than that for fish, 0.0017 µg/L. Mesocosm results presented by Maund et al. (1998) supported these findings, with the reported NOAECs for invertebrate communities and bluegill sunfish of 0.017 and 0.17 µg /L lambda-cyhalothrin, respectively. Authors concluded that adsorption of lambda-cyhalothrin to sediments reduced bioavailability in natural systems and decreased the risk to aquatic communities.

SSDs constructed from acute toxicity values for bifenthrin and permethrin gave approximate HC_{10} values for aquatic communities of 0.02 µg/L and 0.1 µg/L, respectively (Palmquist et al., 2011). The most sensitive species in both cases were *H. azteca* and *Ceriodaphnia dubia*. Dwyer et al. (2005) determined the toxicity of permethrin to 18 threatened and endangered aquatic species. Atlantic sturgeon, shortnose sturgeon, Lahontan cutthroat trout, spotfin chub, and Apache trout were determined to be the five taxa most susceptible to permethrin, with 96-hour LC_{50} values of less than 1.2 to 1.71 µg/L (Dwyer et al., 2005). By way of comparison, the LC_{50} values for the standard laboratory test species rainbow trout (*Oncorhynchus mykiss*), fathead minnow, and sheepshead minnow (*Cyprinodon variegates*) were 3.31, 9.38, and 17.0 µg/L permethrin, respectively. This suggests that some non-standard fish species may be more sensitive to pyrethroid exposure than standard laboratory species. Similarly, species sensitivity distributions also indicated that

saltwater species are significantly more sensitive to the pyrethroid insecticide permethrin than are freshwater species (Maltby et al., 2005).

8.3.2 Community effects in high-tier mesocosm studies

While SSDs can give an estimation of expected effect concentrations, mesocosm studies provide a more realistic assessment of the community-level toxicity of pyrethroid insecticides. Mesocosm experiments not only incorporate realistic exposure scenarios (as opposed to the simplified laboratory exposure systems used to derive toxicity values for SSDs), but are also able to examine the interspecific interactions that can either mediate or exacerbate the effects of pyrethroid exposure (e.g., with predation pressures, interspecific competition for resources, etc.).

Deltamethrin applications to temporary ponds located in the savannahs of West Senegal had immediate and catastrophic, but largely short-term effects, on the resident aquatic invertebrates. Significant numbers of moribund insects were evident during insecticide application, and these were largely predatory hemipterans and coleopterans (Lahr et al., 2000). Conversely, direct applications of cypermethrin at 0.7 g a.i./ha and lambda-cyhalothrin at 0.17 and 1.7 g a.i./ha produced no effect on the composition of emergent insect communities in mesocosm pond systems (Kedwards et al., 1999). The populations of benthic organisms on artificial substrates, however, were altered, and likely attributable to declines in sensitive Gammaridae and Asellidae taxa. Similarly, beta-cyfluthrin (as commercial product Baythroid) applied to artificial lentic mesocosms reduced both copepod and cladoceran populations, but did not affect community diversity indices (Heimbach et al., 1992). Subsequently, while phytoplankton were largely unaffected by the insecticide treatment, certain species exhibited increased abundance, likely as a result of reduced grazing pressures from copepods and cladocerans.

Given the notable effects on behavioral homeostasis, addition of predation pressures is likely to exacerbate the effects of sublethal pyrethroid exposure. Populations of brine shrimp (*Artemia* sp.) exposed to low levels of esfenvalerate (0.04 to 0.08 µg/L) along with simulated predatory pressure were completely eliminated from laboratory microcosms within 32 to 39 days after exposure (Beketov & Liess, 2005). Subsequently, it is possible that multiple ecological pressures (including predation and competition from less sensitive organisms) could cause greater abundance reductions and longer recovery times than observed following insecticide exposures alone. For instance, bifenthrin applications (39 to 287 ng/L) to outdoor mesocosms reduced copepod abundances, but resulted in increased rotifer and algal abundances (Hoagland et al., 1993). Rotifer abundances were also increased following simulated esfenvalerate drift and runoff events, likely as a response to decreased numbers of copepods and cladocerans from toxic pyrethroid effects and fish predation (Webber et al., 1992). Phytoplankton density also increased as a result of decreased grazing pressures from copepods and cladocerans. Smaller bluegill sunfish were present in ponds treated with high esfenvalerate applications (233 g a.i./ha) and authors theorized that this was a result of: 1) reductions in copepod and cladoceran prey and 2) benthic feeding habits of this stage of bluegill causing an increased exposure to sediment-bound esfenvalerate (Webber et al., 1992).

Esfenvalerate applications to an artificial pond system resulted in increased community chlorophyll production, likely a result of reduced grazing pressures, as cladocerans and copepods were reduced (Samsøe-Petersen et al., 2001). Bacterial and algal blooms were

observed following deltamethrin applications, resulting most likely from indirect effects of a reduction in invertebrate grazer populations (Hanson et al., 2007). Conversely, decreases in algal production have been observed following declines in top predator populations. A reduction in bluegill sunfish reproduction following exposure to pulses of either 0.67 or 1.71 µg/L esfenvalerate caused increases in the numbers of resilient zooplankton (Fairchild et al., 1992). This was observed to repress algal biomass, as invertebrate grazing pressures increased.

Although added pyrethroid mass is likely to be rapidly sequestered by organic material and sediments, short-term exposure to dissolved pyrethroids can still result in significant mesocosm effects. Despite rapid measured dissipation (water column half-life of 10.4 ± 2.0 hours), esfenvalerate negatively impacted mesocosm communities at concentrations as low as 0.25 µg/L. Six pulses of 0.25 µg/L esfenvalerate administered every 2 weeks significantly reduced the total abundance of macroinvertebrates (Fairchild et al., 1992). Authors noted that Ephemeroptera, Gastropoda and Diptera were the most sensitive invertebrate taxa. Likewise, two pulsed exposures to esfenvalerate concentrations as low as 0.08 µg/L decreased the number of multiple invertebrate taxa (including copepods, *Hyalella azteca*, and chironomids) up to 54 days after the first exposure. Conversely, numbers of cladocerans and oligochaetes increased following initial population reductions (Lozano et al., 1992). Loss of invertebrates had a positive effect on primary producers, but community metabolism was unaffected by esfenvalerate exposure.

Following pyrethroid exposure, rapid recovery has been observed, although the taxa-specific rates of recovery are variable and likely depend on life history strategies such as generation time and reproductive output. In general, recovery from pyrethroid exposure is likely to be a function of species-specific sensitivity and reproductive rates. For instance, Sherratt et al. (1999) found that recovery times following cypermethrin exposures were longest for the most sensitive taxa and for those with low reproductive rates. Populations of flying, semi-aquatic insects began to re-establish within days of deltamethrin treatment, while abundances of aquatic invertebrates, such as cladocerans and fairy shrimp, were suppressed longer, as recovery was predicated on the number of resting eggs present in the pond environment (Lahr et al., 2000). Similarly, esfenvalerate exposures had negatively impacted zooplankton communities, through reductions in sensitive cladoceran and copepod taxa, but populations recovered in less than 2 weeks following exposure (Fairchild et al., 1992); rotifers were determined to be less sensitive than other zooplankton.

Copepod and cladoceran zooplankton abundances in mesocosm systems were diminished during both simulated lambda-cyhalothrin runoff and spray drift events (maximum water concentration = 98 ng/L), but quickly rebounded following cessation of insecticide applications. Conversely, rotifers and total macroinvertebrates were increased during the dosing events (Hill et al., 1994). Simulated tralomethrin runoff (218.9 ng/L) and drift (68.5 ng/L) also did not produce any lasting effects on mesocosm ecosystems. Although densities of Caenidae mayflies and copepods were reduced, other organisms, including mollusks, fish, macrophytes, algae, and benthic invertebrates were unaffected (Mayasich et al., 1994).

Examination of the results from multiple mesocosm studies indicates that 1) a certain subset of species can be expected to be most sensitive to pyrethroid insecticides, and 2) community function is rarely, if ever, altered by pyrethroid exposure, although the community structure may be altered. For example, mesocosm community metabolism, as measured by photosynthetic and respiration rates, was unaffected by lambda-cyhalothrin treatment, despite alterations in the invertebrate community (Hill et al., 1994). Giddings et al. (2001)

analyzed the results of seven different pyrethroid mesocosm studies and determined that amphipods, copepods, cladocerans, mayflies, caddisflies and midges were consistently the most sensitive invertebrates. However, reductions in amphipod and isopods were accompanied by increases in less sensitive organisms that occupy similar ecological niches (Giddings et al., 2001). Although pyrethroids were typically associated with sediment after introduction into mesocosms, the population- and community-level impacts observed in exposed mesocosms were correlated most closely to maximum water concentrations (Giddings et al., 2001). Recovery of affected species was determined to be governed by species-specific attributes, such as generation time and reproductive rate. Zooplankton populations, for instance, recovered quickly, as they have short generation times and high reproductive output (Giddings et al., 2001). In addition to factors that influence recovery rate, other life history strategies are likely to alter the effects of pyrethroid exposure on aquatic species. Zooplankton resting egg stages located in sediments likely comprised a significant ecological reservoir, for which re-colonization following pyrethroid exposure can occur (Hanson et al. 2007). In addition, some species may inhabit microenvironments that are refugia from pyrethroid exposures, and those species with efficient or rapid immigration, are more likely to recover rapidly from pyrethroid exposures (Giddings et al., 2001).

9. Conclusions

The usage of pyrethroid insecticides increased as a response to the phasing out of other insecticide classes. In contrast to other insecticides, pyrethroids exhibit lower mammalian and avian toxicity and better selectivity to target species than OPs and less persistence than organochlorine insecticides.

Drift and runoff from sprayed outdoor areas are probably the primary routes of pyrethroid movement into nontarget aquatic and terrestrial environments. Indoor use of pyrethroids is expected to be a very small component of total applications, and therefore is unlikely to result in significant environmental concentrations. Pyrethroids do not readily volatilize and exhibit strong adsorption to organic matter, soils and clay, an aspect that largely governs their environmental fate and bioavailability.

Although pyrethroid insecticides were developed for the control of terrestrial pest insects, there is evidence that nontarget aquatic invertebrate species may be more sensitive than terrestrial invertebrates. Pyrethroids have also been demonstrated to be toxic to nontarget aquatic vertebrates, such as fish and amphibians. However, evidence from aquatic mesocosm studies suggests that adverse population- and community-level effects following pyrethroid exposure are probably short-term, and that recovery from these is rapid. In terrestrial systems, spray drift generally results in non-homogenous distribution of pyrethroids in edge-of-field and off-crop areas. Hence, impacts on nontarget invertebrates in these areas are spatially variable, and recovery can occur via immigration from non-impacted populations.

As pyrethroids provide effective control of pest insects, widespread use is expected to continue. In order to better understand the environmental impacts of this use, exposure and effects studies that incorporate environmentally relevant concentrations should continue to be conducted. In addition, research into best management practices, such as using vegetation buffers or timing applications to avoid exposure to nontarget organisms, or development of formulations that are less likely to be transported into nearly streams and

rivers, should be conducted. It is also imperative that results of that research be conveyed to the public and private sectors so that the professional pest applicators and private homeowners who use these products around their homes do so in an environmentally-friendly manner.

10. References

Åkerblom, N., Arbjörk, C., Hedlund, M. and Goedkoop, W. (2008). Deltamethrin toxicity to the midge Chironomus riparius Meigen--Effects of exposure scenario and sediment quality. *Ecotoxicology and Environmental Safety* 70, 53-60.

Ali SFM, Shieh BHH, Alehaideb Z, Khan MZ, Louie A, Fageh N, Law FCP. (2011). A review on the effects of some selected pyrethroids and related agrochemicals on aquatic vertebrate biodiversity. *Canadian Journal of Pure and Applied Science* 5(2): 1455-1464.

Amweg, E. L., Weston, D. P. and Ureda, N. M. (2005). Use and toxicity of pyrethroid pesticides in the Central Valley, California, USA. *Environmental Toxicology and Chemistry* 24, 966-972.

Anderson, R. L. (1982). Toxicity of Fenvalerate and Permethrin to Several Nontarget Aquatic Invertebrates. *Environmental Entomology* 11, 1251-1257.

Arts, G. H. P., Buijse-Bogdan L. L., Belgers J. D. M., Rhenen-Kersten C. H. , Van Wijngaarden, R. P. A. ,Van Roessink I., Maund S. J., Brink P. J. van den, Brock, T. C. M. (2006). Ecological impact in ditch mesocosms of simulated spray drift from a crop protection program for potatoes. *Integrated Environmental Assessment and Management (IEAM)* 2, 105-125.

Beketov, M. A. and Liess, M. (2005). Acute contamination with esfenvalerate and food limitation: chronic effects on the mayfly, Cloeon dipterum. *Environmental Toxicology and Chemistry* 24, 1281-1286.

Bennett, E. R., Moore, M. T., Cooper, C. M., Smith, S., Shields, F. D., Drouillard, K. G. and Schulz, R. (2005). Vegetated agricultural drainage ditches for the mitigation of pyrethroid-associated runoff. *Environmental Toxicology and Chemistry* 24, 2121-2127.

Berrill, M., Bertram, S., Wilson, A., Louis, S., Brigham, D. and Stromberg, C. (1993). Lethal and sublethal impacts of pyrethroid insecticides on amphibian embryos and tadpoles. *Environmental Toxicology and Chemistry* 12, 525-539.

Boone, M. D. (2008). Examining the single and interactive effects of three insecticides on amphibian metamorphosis. *Environmental Toxicology and Chemistry* 27, 1561-1568.

Brooks, J. E., Savarie, P. J. and Bruggers, R. L. (1998a). The toxicity of commercial insecticide aerosol formulations to brown tree snakes. *Snake* 28, 23-27.

Chapman, R. A., Tu, C. M., Harris, C. R. and Cole, C. (1981). Persistence of five pyrethroid insecticides in sterile and natural, mineral and organic soil. *Bulletin of environmental contamination and toxicology* 26, 513-519.

Cold, A. and Forbes, V. E. (2004). Consequences of a short pulse of pesticide exposure for survival and reproduction of *Gammarus pulex*. *Aquatic Toxicology* 67, 287-299.

Cox, R. L. and Wilson, W. T. (1984). Effects of permethrin on the behavior of individually tagged honey bees, *Apis mellifera* L. (Hymenoptera: Apidae). *Environmental Entomology* 13, 375-378.

Cripe, G. M. (1994). Comparative acute toxicities of several pesticides and metals to *Mysidopsis bahia* and postlarval *Penaeus duorarum*. *Environmental Toxicology and Chemistry* 13, 1867-1872.

Dai, P.-L., Wang, Q., Sun, J.-H., Liu, F., Wang, X., Wu, Y.-Y. and Zhou, T. (2010). Effects of sublethal concentrations of bifenthrin and deltamethrin on fecundity, growth, and development of the honeybee *Apis mellifera ligustica*. *Environmental Toxicology and Chemistry* 29, 644-649.

Decourtye, A., Devillers, J., Genecque, E., Menach, K. L., Budzinski, H., Cluzeau, S. and Pham-Delègue, M. H. (2005). Comparative sublethal toxicity of nine pesticides on olfactory learning performances of the honeybee *Apis mellifera*. *Archives of environmental contamination and toxicology* 48, 242-250.

Delpuech, J.-M., Legallet, B., Terrier, O. and Fouillet, P. (1999). Modifications of the sex pheromonal communication of *Trichogramma brassicae* by a sublethal dose of deltamethrin. *Chemosphere* 38, 729-739.

DeMicco, A., Cooper, K. R., Richardson, J. R. and White, L. A. (2010). Developmental neurotoxicity of pyrethroid insecticides in zebrafish embryos. *Toxicological Sciences* 113, 177-186.

Desneux, N., Decourtye, A. and Delpuech, J. M. (2007). The sublethal effects of pesticides on beneficial arthropods. *Annual Review of Entomology* 52, 81-106.

Dwyer, F. J., Mayer, F. L., Sappington, L. C., Buckler, D. R., Bridges, C. M., Greer, I. E., Hardesty, D. K., Henke, C. E., Ingersoll, C. G., Kunz, J. L. et al. (2005). Assessing contaminant sensitivity of endangered and threatened aquatic species: Part I. acute toxicity of five chemicals. *Archives of environmental contamination and toxicology* 48, 143-154.

Ecobichon D.J. (1996). Toxic effects of pesticides. In: *Casarett & Doull's Toxicology: The Basic Science of Poisons*, Klaassen C.D., Amdur MO, Doull J. (Eds). McGraw-Hill, Health Professions Division. New York, NY. 1996. 643-690.

Fairchild, J. F., La Point, T. W., Zajicek, J. L., Nelson, M. K., Dwyer, F. J. and Lovely, P. A. (1992). Population-, community- and ecosystem-level responses of aquatic mesocosms to pulsed doses of a pyrethroid insecticide. Environmental Toxicology and Chemistry.11, 115-129.

Feo, M.L., Eljarrat, E., Barcelo', D. (2010). Determination of pyrethroid insecticides in environmental samples. *Trends in Analytical Chemistry* 29(7):692-705.

Fishel FM. 2005. Pesticide Toxicity Profile: Synthetic Pyrethroid Pesticides. Agronomy Department, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida. Report #PI-54.

Fleming, R. J., Holmes, D. and Nixon, S. J. (1998). Toxicity of permethrin to *Chironomus riparius* in artificial and natural sediments. *Environmental Toxicology and Chemistry* 17, 1332-1337.

Floyd, E. Y., Geist, J. P. and Werner, I. (2008). Acute, sublethal exposure to a pyrethroid insecticide alters behavior, growth, and predation risk in larvae of the fathead minnow (Pimephales promelas). *Environmental Toxicology and Chemistry* 27, 1780-1787.

Friesen, K. J. and Kaufman, W. R. (2003). Cypermethrin inhibits egg development in the ixodid tick, *Amblyomma hebraeum*. *Pesticide Biochemistry and Physiology* 76, 25-35.

Gan, J., Lee, S. J., Liu, W. P., Haver, D. L. and Kabashima, J. N. (2005). Distribution and persistence of pyrethroids in runoff sediments. *J. Environ. Qual.* 34, 836-841.

Giddings, J. M., Solomon, K. R. and Maund, S. J. (2001). Probabilistic risk assessment of cotton pyrethroids: II. Aquatic mesocosm and field studies. *Environmental Toxicology and Chemistry* 20, 660-668.

González-Doncel, M., de la Peña, E., Barrueco, C. and Hinton, D. E. (2003). Stage sensitivity of medaka (*Oryzias latipes*) eggs and embryos to permethrin. *Aquatic Toxicology* 62, 255-268.

Greulich, K. and Pflugmacher, S. (2003). Differences in susceptibility of various life stages of amphibians to pesticide exposure. *Aquatic Toxicology* 65, 329-336.

Hall, F. R. (1979). Effects of Synthetic Pyrethroids on Major Insect and Mite Pests of Apple. *Journal of economic entomology* 72, 441-446.

Hammond, R. B. (1996). Residual activity of lambda-cyhalothrin against bean leaf beetle (Coleoptera: Chrysomelidae) in soybeans. *Journal of Agricultural Entomology* 13, 365-373.

Hanson, M. L., Graham, D. W., Babin, E., Azam, D., Coutellec, M.-A., Knapp, C. W., Lagadic, L. and Caquet, T. (2007). Influence of isolation on the recovery of pond mesocosms from the application of an insecticide. I. Study design and planktonic community responses. *Environmental Toxicology and Chemistry* 26, 1265-1279.

Haynes, K. F. (1988). Sublethal effects of neurotoxic insecticides on insect behavior. *Annual Review of Entomology* 33, 149-168.

Haynes, K. F. and Baker, T. C. (1985). Sublethal effects of permethrin on the chemical communication system of the pink bollworm moth, *Pectinophora gossypiella*. *Archives of Insect Biochemistry and Physiology* 2, 283-293.

Heimbach, F., Pflueger, W. and Ratte, H.-T. (1992). Use of small artificial ponds for assessment of hazards to aquatic ecosystems. *Environmental Toxicology and Chemistry* 11, 27-34.

Hill, I. R., Runnalls, J. K., Kennedy, J. H. and Ekoniak, P. (1994a). Lambda-cyhalothrin: A mesocosm study of its effects on aquatic organisms. In *Aquatic Mesocosm Studies in Ecological Risk Assessment*, eds. R. L. Graney J. H. Kennedy and J. H. Rodgers Jr.). Boca Raton, Fl: Lewis Publishers.

Hoagland, K. D., Drenner, R. W., Smith, J. D. and Cross, D. R. (1993). Freshwater community responses to mixtures of agricultural pesticides: Effects of atrazine and bifenthrin. *Environmental Toxicology and Chemistry* 12, 627-637.

Hull, L. A., Beers, E. H. and Meagher, R. L. (1985). Integration of Biological and Chemical Control Tactics for Apple Pests Through Selective Timing and Choice of Synthetic Pyrethroid Insecticides. *Journal of economic entomology* 78, 714-721.

Inglesfield, C. (1989). Pyrethroids and terrestrial nontarget organisms. *Pesticide science* 27, 387-428.

Ishaaya, I., Elsner, A., Ascher, K. R. S. and Casida, J. E. (1983). Synthetic pyrethroids: Toxicity and synergism on dietary exposure of *Tribolium castaneum* (Herbst) larvae. *Pesticide science* 14, 367-372.

Jin, H. and Webster, G. R. B. (1998). Persistence, Penetration, and Surface Availability of Cypermethrin and Its Major Degradation Products in Elm Bark. *Journal of Agricultural and Food Chemistry* 46, 2851-2857.

Jin, M., Zhang, X., Wang, L., Huang, C., Zhang, Y. and Zhao, M. (2009). Developmental toxicity of bifenthrin in embryo-larval stages of zebrafish. *Aquatic Toxicology* 95, 347-354.

Johansson, M., Piha, H., Kylin, H. and Merilä, J. (2006). Toxicity of six pesticides to common frog (*Rana temporaria*) tadpoles. *Environmental Toxicology and Chemistry* 25, 3164-3170.

Johnson, K. R., Jepson, P. C. and Jenkins, J. J. (2008). Esfenvalerate-induced case-abandonment in the larvae of the caddisfly (Brachycentrus americanus). *Environmental Toxicology and Chemistry* 27, 397-403.

Juarez, M. P. (1995). The effect of sublethal doses of insecticides on *Triatoma infestans* lipid synthesis. *Pesticide Biochemistry and Physiology* 52, 81-89.

Katagi, T. (1991). Photodegradation of the pyrethroid insecticide esfenvalerate on soil, clay minerals, and humic acid surfaces. *Journal of Agricultural and Food Chemistry* 39, 1351-1356.

Kaufman, D. D., Russell, B. A., Helling, C. S. and Kayser, A. J. (1981). Movement of cypermethrin, decamethrin, permethrin, and their degradation products in soil. *Journal of Agricultural and Food Chemistry* 29, 239-245.

Kedwards, T. J., Maund, S. J. and Chapman, P. F. (1999). Community level analysis of ecotoxicological field studies: II. Replicated-design studies. *Environmental Toxicology and Chemistry* 18, 158-166.

Lahr, J., Diallo, A. O., Gadji, B., Diouf, P. S., Bedaux, J. J. M., Badji, A., Ndour, K. B., Andreasen, J. E. and van Straalen, N. M. (2000). Ecological effects of experimental insecticide applications on invertebrates in sahelian temporary ponds. *Environmental Toxicology and Chemistry* 19, 1278-1289.

Langhof, M., Gathmann, A., Poehling, H. M., Vogt, H., Heimbach, U. and Vinuela, E. (2003). Field study on the effects of insecticide drift on nontarget arthropods : residual toxicity, effect on populations and recolonisation processes. *Bulletin OILB/SROP* 26, 73-82.

Lee, C. Y., Yap, H. H. and Chong, N. L. (1998). Sublethal effects of deltamethrin and propoxur on longevity and reproduction of German cockroaches, *Blattella germanica*. *Entomologia Experimentalis et Applicata* 89, 137-145.

Lee, P. W. (1985). Fate of fenvalerate (Pydrin insecticide) in the soil environment. *Journal of Agricultural and Food Chemistry* 33, 993-998.

Little, E. E., Dwyer, F. J., Fairchild, J. F., Delonay, A. J. and Zajicejk, J. L. (1993). Survival of bluegill and their behavioral responses during continuous and pulsed exposures to esfenvalerate, a pyrethroid insecticide. *Environmental Toxicology and Chemistry* 12, 871-878.

Liu, W., Gan, J. J., Lee, S. and Kabashima, J. N. (2004). Phase distribution of synthetic pyrethroids in runoff and stream water. *Environmental Toxicology and Chemistry* 23, 7-11.

Longley, M. (1999). A review of pesticide effects upon immature aphid parasitoids within mummified hosts. *International Journal of Pest Management* 45, 139 - 145.

Longley, M. and Jepson, P. C. (1996). Effects of honeydew and insecticide residues on the distribution of foraging aphid parasitoids under glasshouse and field conditions. *Entomologia Experimentalis et Applicata* 81, 189-198.

Lozano, S. J., O'Halloran, S. L., Sargent, K. W. and Brazner, J. C. (1992). Effects of esfenvalerate on aquatic organisms in littoral enclosures. *Environmental Toxicology and Chemistry* 11, 35-47.

Luo, Y., and Zhang, M. (2011). Environmental modeling and exposure assessment of sediment-associated pyrethroids in an agricultural watershed. PLoS ONE 6(1):e15794. Doi:10.1371/journal.pone.0015794.

Maltby, L., Blake, N., Brock, T. C. M. and Van den Brink, P. J. (2005). Insecticide species sensitivity distributions: Importance of test species selection and relevance to aquatic ecosystems. *Environmental Toxicology and Chemistry* 24, 379-388.

Materna, E. J., Rabeni, C. F. and Lapoint, T. W. (1995). Effects of the synthetic pyrethroid insecticide, esfenvalerate, on larval leopard frogs (*Rana* spp.). *Environmental Toxicology and Chemistry* 14, 613-622.

Maul, J. D., Brennan, A. A., Harwood, A. D. and Lydy, M. J. (2008a). Effect of sediment-associated pyrethroids, fipronil, and metabolites on Chironomus tentans growth rate, body mass, condition index, immobilization, and survival. *Environmental Toxicology and Chemistry* 27, 2582-2590.

Maul, J. D., Trimble, A. J. and Lydy, M. J. (2008b). Partitioning and matrix-specific toxicity of bifenthrin among sediments and leaf-sourced organic matter. *Environmental Toxicology and Chemistry* 27, 945-952.

Maund, S. J., Hamer, M. J., Warinton, J. S. and Kedwards, T. J. (1998). Aquatic ecotoxicology of the pyrethroid insecticide lambda-cyhalothrin: considerations for higher-tier aquatic risk assessment. *Pesticide science* 54, 408-417.

Maund, S. J., Travis K Z, Hendley P, Giddings J M and Solomon K R. (2001). Probabilistic risk assessment of cotton pyrethroids: V. Combining landscape-level exposures and ecotoxicological effects data to characterize risks. *Environmental Toxicology and Chemistry* 20, 687-692.

Mayasich, J. M., Kennedy, J. H. and O'Grodnick, J. S. (1994). Evaluation of the ecological and biological effects of tralomethrin utilizing an experimental pond system. In *Aquatic Mesocosm Studies in Ecological Risk Assessment*, eds. R. L. Graney J. H. Kennedy and J. H. Rodgers Jr.). Boca Raton, FL: Lewis Publishers.

Moore, A. and Waring, C. P. (2001). The effects of a synthetic pyrethroid pesticide on some aspects of reproduction in Atlantic salmon (*Salmo salar* L.). *Aquatic Toxicology* 52, 1-12.

Moore, M.T., Cooper, C.M., Smith, S., Cullum, R.F., Knight, S.S., Locke, M.A., Bennett, E.R. 2009. Mitigation of two pyrethroid insecticides in a Mississippi Delta constructed wetland. *Environmental Pollution* 157:250-256.

Moreau, R., Carle, P. R., Gourdoux, L., Couillaud, F., Khay, A. B. and Girardie, A. (1987). Deltamethrin: In vivo effects on glucose catabolism in *Locusta migratoria* L. *Pesticide Biochemistry and Physiology* 27, 101-105.

Morolli, C., Quaglio, F., Della Rocca, G., Malvisi, J. and Di Salvo, A. (2006). Evaluation of the Toxicity of Synthetic Pyrethroids to Red Swamp Crayfish (Procambarus clarkii, Girard 1852) and Common Carp (Cyprinus carpio, L. 1758). *Bull. Fr. Pêche Piscic.* 380-381, 1381-1394.

Muir, D. C. G., Rawn, G. P., Townsend, B. E., Lockhart, W. L. and Greenhalgh, R. (1985). Bioconcentration of cypermethrin, deltamethrin, fenvalerate and permethrin by *Chironomus tentans* larvae in sediment and water. *Environmental Toxicology and Chemistry* 4, 51-61.

Oros, D. R. and Werner, I. (2005). Pyrethroid Insecticides: An Analysis of Use Patterns, Distributions, Potential Toxicity and Fate in the Sacramento-San Joaquin Delta and

Central Valley. In *White Paper for the Interagency Ecological Program*, vol. SFEI Contribution 415. Oakland, CA: San Francisco Estuary Institute.

Palmquist, K., Fairbrother, A., Salatas, J. and Guiney, P. D. (2011). Environmental fate of pyrethroids in urban and suburban stream sediments and the appropriateness of Hyalella azteca model in determining ecological risk. *Integrated Environmental Assessment and Management* 7, 325-335.

Palmquist, K. R., Jenkins, J. J. and Jepson, P. C. (2008a). Effects of dietary esfenvalerate exposures on three aquatic insect species representing different functional feeding groups. *Environmental Toxicology and Chemistry* 27, 1721-1727.

Palmquist, K. R., Jepson, P. C. and Jenkins, J. J. (2008b). Impact of aquatic insect life stage and emergence strategy on sensitivity to esfenvalerate exposure. *Environmental Toxicology and Chemistry* 27, 1728-1734.

Pap, L., Bajomi, D., and Szekely, I. (1996). The pyrethroids, an overview. *International Pest Council* 38(1):15-19.

Posthuma, L., Suter, G.W. II. and Traas, T.P. (2002). Species Sensitivity Distributions in Ecotoxicology. CRC Press. Boca Raton, FL. 616pp.,

Ranson, H., N'Guessan, R, Lines, J., Moiroux, N., Nkuni, Z., and Corbel, V. (2011). Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? *Trends in Parasitology* 27(2):91-98.

Ray, D.E. (2000). Pesticide neurotoxicity in Europe: real risks and perceived risks. *NeuroToxicology* 21(1-2):219-222.

Reynaldi, S., Duquesne, S., Jung, K. and Liess, M. (2006). Linking feeding activity and maturation of Daphnia magna following short - term exposure to fenvalerate. *Environmental Toxicology and Chemistry* 25, 1826-1830.

Reynaldi, S. and Liess, M. (2005). Influence of duration of exposure to the pyrethroid fenvalerate on sublethal responses and recovery of Daphnia magna straus. *Environmental Toxicology and Chemistry* 24, 1160-1164.

Rice, P. J., Drewes, C. D., Klubertanz, T. M., Bradbury, S. P. and Coats, J. R. (1997). Acute toxicity and behavioral effects of chlorpyrifos, permethrin, phenol, strychnine, and 2,4-dinitrophenol to 30-day-old Japanese medaka (Oryzias latipes). *Environmental Toxicology and Chemistry* 16, 696-704.

Samsøe-Petersen, L., Gustavson, K., Madsen, T., Mogensen, B. B., Lassen, P., Skjernov, K., Christoffersen, K. and Jørgensen, E. (2001). Fate and effects of esfenvalerate in agricultural ponds. *Environmental Toxicology and Chemistry* 20, 1570-1578.

Schulz, R. and Liess, M. (2000). Toxicity of fenvalerate to caddisfly larvae: chronic effects of 1- vs 10-h pulse-exposure with constant doses. *Chemosphere* 41, 1511-1517.

Sherratt, T. N., Roberts, G., Williams, P., Whitfield, M., Biggs, J., Shillabeer, N. and Maund, S. J. (1999). A life-history approach to predicting the recovery of aquatic invertebrate populations after exposure to xenobiotic chemicals. *Environmental Toxicology and Chemistry* 18, 2512-2518.

Soderlund DM, Bloomquist JR.1989. Neurotoxic actions of pyrethroid insecticides. *Ann. Rev. Entomol.* 34:77-96.

Srivastava, R. K., Yadav, K. K. and Trived, S. P. (2008). Devicyprin induced gonadal impairment in a freshwater food fish, Channa punctatus (Bloch). *Journal of Environmental Biology* 29, 187-191.

Stueckle, T. A., Shock, B. and Foran, C. M. (2009). Multiple stressor effects of methoprene, permethrin, and salinity on limb regeneration and molting in the mud fiddler crab (UCA pugnax). *Environmental Toxicology and Chemistry* 28, 2348-2359.

Talent, L. G. (2005). Effect of temperature on toxicity of a natural pyrethrin pesticide to green anole lizards (Anolis carolinensis). *Environmental Toxicology and Chemistry* 24, 3113-3116.

Vandame, R., Meled, M., Colin, M.-E. and Belzunces, L. P. (1995). Alteration of the homing-flight in the honey bee *Apis mellifera* L. exposed to sublethal dose of deltamethrin. *Environmental Toxicology and Chemistry* 14, 855-860.

US EPA. (2000). Chlorpyrifos revised risk assessment and agreement with registrants. Washington, D.C.: U.S. Environmental Protection Agency.

US EPA. (2001). Diazinon revised risk assessment and agreement with registrants. Prevention, Pesticides and Toxic Substances. Washington, D.C.: U.S. Environmental Protection Agency.

Walker, K. (2000). Cost-comparison of DDT and alternative insecticides for malaria control. *Medical and Veterinary Entomology* 14:354-354.

Webber, E. C., Deutsch, W. G., Bayne, D. R. and Seesock, W. C. (1992). Ecosystem-level testing of a synthetic pyrethroid insecticide in aquatic mesocosms. *Environmental Toxicology and Chemistry* 11, 87-105.

Weston DP, Lydy MJ. 2010. Urban and Agricultural Sources of Pyrethroid Insecticides to the Sacramento-San Joaquin Delta of California. Environ. Sci. Technol. 44(5): 1833-1840.

Wiles, J. A. and Jepson, P. C. (1994). Substrate-mediated toxicity of deltamethrin residues to beneficial invertebrates: Estimation of toxicity factors to aid risk assessment. *Archives of environmental contamination and toxicology* 27, 384-391.

WHOPES. (2005). Safety of pyrethroids for public health use. WHO Pesticide Evaluation Scheme.

World Health Organization. (1989a). *Environmental Health Criteria 92 – Resmethrin*. International Programme on Chemical Safety, World Health Organization: Geneva, Switzerland, pp 1-79.

World Health Organization. (1989b). *Environmental Health Criteria 82 – Cypermethrin*. International Programme on Chemical Safety, World Health Organization: Geneva, Switzerland, pp 1-88.

World Health Organization. (1990a). *Environmental Health Criteria 96 – d-Phenothrin*. International Programme on Chemical Safety, World Health Organization: Geneva, Switzerland, pp 1-33.

World Health Organization. (1990b). *Environmental Health Criteria 94 - Permethrin*. International Programme on Chemical Safety, World Health Organization: Geneva, Switzerland, pp 1-58.

You, J., Pehkonen, S., Weston, D. P. and Lydy, M. J. (2008). Chemical availability and sediment toxicity of pyrethroid insecticides to Hyalella azteca: Application to field sediment with unexpectedly low toxicity. *Environmental Toxicology and Chemistry* 27, 2124-2130.

12

Hepatic Effects from Subacute Exposure to Insecticides in Adult Male Wistar Rats

María-Lourdes Aldana-Madrid et al.[1]
Departamento de Investigación y Posgrado en Alimentos,
Universidad de Sonora Hermosillo,
Sonora,
México

1. Introduction

Pesticides are applied to grains during growth, post harvest, and storage, in order to prevent pest infestation that could damage the crops integrity and affect harvest. Recent studies have shown that insecticide residues are present in grains and other foodstuffs (wheat, corn, beans, and chickpeas) stored in Sonora, Mexico (Aldana et al, 2008). Concentrations of the most common insecticides such as DDT were detected to be within safe levels for consumption as established by the World Health Organization (Food and Agriculture Organization of the United Nations [FAO/WHO], 1999), although the use of some detected insecticides such as DDT and metabolites are no longer permitted in Mexico (CICOPLAFEST, 2004). Most grains are milled prior to human consumption. However, processing does not completely eliminate the insecticide (Pimentel & Casadei, 2000; Holland et al., 1994). In Mexico, residues of organochlorine (OC) insecticides are detected in adipose tissue, blood, and breast milk in populations living near agricultural fields. Studies document OC exposures in Comarca Lagunera, Mexico City, Puebla, Veracruz (RAPAM, 1997) and the Yaqui Valley in Sonora (Guillette et al., 1998). Such exposures are of great concern, especially since exposure to insecticides has been shown to alter lipid metabolism and produce liver damage in rats (Shakoori et al., 1998; Narayan et al., 1990; Videira et al., 2001; Aldana et al., 1998; Aldana et al., 2001).

The objective of our study was to evaluate the potential hepatotoxic effects of insecticide exposure at these levels in rats, using serum biochemical indicators of hepatic damage (transaminases, cholesterol, triglycerides, total protein, and albumin), as well as light and electron microscopy to evaluate microstructure hepatic changes.

---

[1]Mineko Shibayama[2], Margarita Calderon[1], Angélica Silva[2], María-Isabel Silveira-Gramont[1], Víctor Tsutsumi[2], Fabiola-Gabriela Zuno-Floriano[3] and Ana-Rosa Rincón-Sánchez[4]
1 Departamento de Investigación y Posgrado en Alimentos, Universidad de Sonora. Hermosillo, Sonora, México
2 Departamento de Infectómica y Patogénesis Molecular, CINVESTAV-IPN, México D.F. México
3 Department of Environmental Toxicology, University of California, Davis, California, USA
4 Departamento de Biología Molecular y Genómica, CUCS. Universidad de Guadalajara, Guadalajara, Jalisco, México

2. Materials and methods

2.1 Materials

Pluronic F-68 (20% purity) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Malathion (98.5% purity), chlorpyriphos (99.6% purity), and deltamethrin (99.8% purity) were obtained from Sigma-Aldrich (SAF, Deisenhofen, Germany). CYP (91% purity; *cis/trans*, 49.9:50.1) was from Zeneca Co. (ICI of Mexico) and 4,4'-DDT (99% purity) was from Chem Service (West Chester, PA, USA).

2.2 Animals

Seventy-six male Wistar rats were purchased from the University of Puebla farm, (Mexico), weighing 280–300 g and were acclimated for a minimum of 7 days before treatment. The animals were randomly selected, based on their body weight and housed in individual stainless-steel wire hanging cages during the 7 days of treatment. They were exposed to a 12-h light/dark cycle, at a room temperature of 19–22°C. The animals had free access to a rodent lab diet (Ralston Rations, Purina, St. Louis, MO) and water. Rats were divided into seven groups as shown in figure 1.

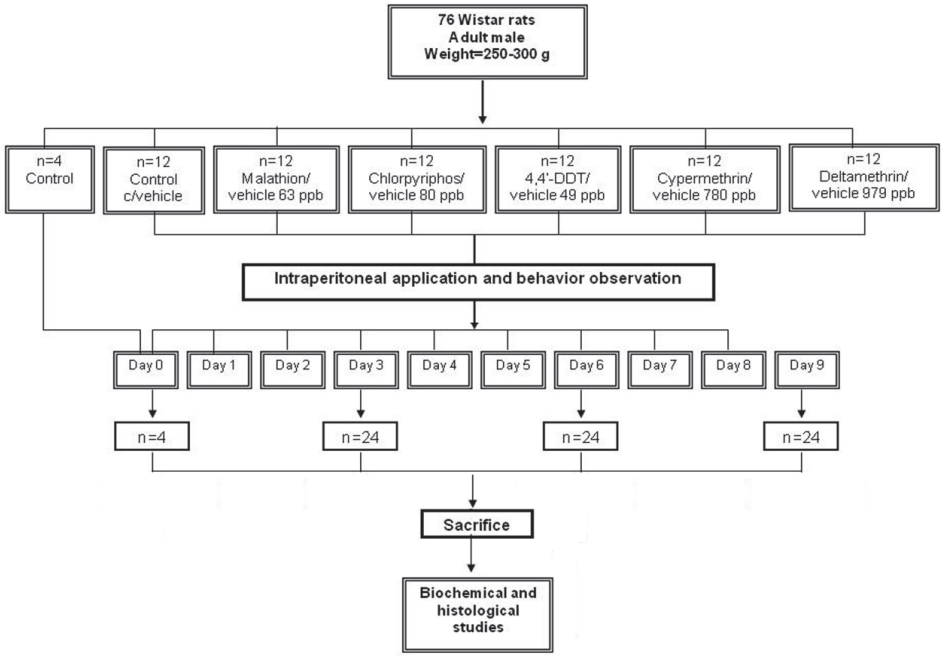


Fig. 1. Study outline. Adult male Wistar rats were injected IP with insecticides for 9 consecutive days, Malathion, 63 μg kg^{-1}; Chlorpyriphos, 80 μg kg^{-1}; Cypermethrin, 780 μg kg^{-1}; Deltamethrin, 979 μg kg^{-1}; and 4,4'-DDT 49 μg kg^{-1}. In the insecticide-treated groups 12 animals were included, and in the pluronic control group (administered with Pluronic F-68 as vehicle). Animals were sacrificed on days 0, 3, 6 and 9. The effects on serum levels of cholesterol, triglycerides, total protein, albumin, and hepatic enzymes alanine aminotransferase and aspartate aminotransferase were measured at each time point. Changes in liver cell morphology were also evaluated.

One group was sacrificed after the seven-day adjustment period to light/food (day 0), and was free of any exposure. The second group only received pluronic F-68 (20%) as a vehicle for nine consecutive days (Aldana et al., 1998; Aldana et al., 2001). The five experimental groups were exposed by intraperitoneal injection (IP) for nine consecutive days to malathion (MAL), chlorpyriphos (CHL), cypermethrin (CYP), deltamethrin (DEL) and 4,4'-DDT at dose of 63 μg kg^{-1}, 80 μg kg^{-1}, 780 μg kg^{-1}, 979 μg kg^{-1}, and 49 μg kg^{-1} body weight per day, respectively. The doses used in the present study were similar to those that induce subacute intoxication and were similar to concentrations detected in stored grains in a previous work[1]. Permitted residual concentrations published in CICOPLAFEST (2004) and concentrations used in this study were included in table 1. Pesticides were administrated by IP injection to assure the total exposure to each insecticide. The side of administration is very close to the liver which is the target organ in this study.
At three-day intervals, four rats from each group, including the pluronic control group, were sacrificed (days 3, 6, 9).

INSECTICIDES PERMITTED						**NO PERMITTED**
Concentration	**Malathion**	**Chlorpyriphos**		**Deltamethrin**	**Cypermethrin**	**4,4'-DDT**
Permitted (mg/kg)	8.0	10.0		1.0	0.05	----
Found in grains (mg/kg)	0.063	0.080		0.979	0.78	0.049
Grains	Bean	Corn		Bean	Bean, corn	

Table 1. Insecticides concentrations permitted and concentration found in grains in Sonora State.

At 3-day intervals, 4 rats from each group were sacrificed (days 3, 6, 9). Food was removed 12 h prior to sacrifice. Blood was collected by cardiac puncture while rats were under ether anesthesia. The animals were euthanized using an overdose of anesthetics.
The present study complied with the institution's guidelines and the Mexican official regulation (NOM-062-ZOO-1999) regarding technical specifications for production, care, and use of laboratory animals. The protocol was also approved by the local animal ethics committee.

2.3 Behavioral and physical evaluation

Drowsiness, bristly hair, pruritus and aggressiveness were assessed and recorded as signs of toxicity after insecticide exposure. Animals were observed daily. The animals in each group were weighed daily and prior to sacrifice.

2.4 Biochemical assays

Blood samples were collected at pre-determined time points and centrifuged at 800×g for 3 minutes. Serum samples were also collected, and used in the following assays: alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, cholesterol, triglycerides (TG), total proteins, and albumin concentration analysis (Figure 3). These assays were performed with the Monotest^{MR} Lakeside (Boehringer Mannheim GmbH,

Seelze, Germany) and a Hitachi 912 automated instrument spectrophotometer from the Clinical Analysis Laboratory at the Sonora State General Hospital, according to the specifications of the International Foundation of Clinical Chemistry (IFCC, 1986).

2.5 Light and electron microscopy studies

Immediately after euthanizing the animals, livers were removed and representative tissue fragments were placed in 2.5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA, USA) in 0.1M sodium cacodylate buffer, pH 7.2 (Electron Microscopy Sciences, Fort Washington, PA, USA), and processed for embedding in epoxy resin. Sections 0.5 µm thick were stained with toluidine blue and examined by light microscopy (Nikon, Co. Japan). Thin sections were contrasted with uranyl acetate and lead citrate and examined by transmission electron microscopy (Jeol 100SX, Japan).

3. Statistical analysis

Analyses of variance (ANOVA) and linear regression were performed to determine the statistical significance of the effects of pesticides on the biochemical and histological indicators of liver function. All statistical analyses were carried out using JMP version 4.

4. Results

4.1 Behavioral and physical evaluation

Among the insecticides chosen for this study, MAL is classified as slightly hazardous (class III), and CHL, CYP, DEL and 4,4'-DDT are classified as to moderate hazardous (class II) as shown in table 2 (International Program of the Chemical Safety [IPCS], 2009).

The sings of toxicity in the animals were related to the concentration of insecticides used. The animals treated with slight or moderately toxic insecticides showed a higher degree of drowsiness. Bristly hair, and pruritus were present in all treated groups, but a higher percent was evident in the moderately toxic compounds. Aggressiveness was mainly noticed in some of the moderately toxic compounds.

Insecticide	Class*	Toxicity	Drowsiness (%)	Bristly Hair (%)	Itching (%)	Aggressiveness (%)
Control		none	0	0	0	0
Malathion	III	slightly hazardous	58	35	38	0
Chlorpyriphos	II	Moderately hazardous	60	28	28	6
Cypermethrin	II	Moderately hazardous	1	50	38	13
Deltamethrin	II	Moderately hazardous	0	56	35	35
4,4'-DDT	II	Moderately hazardous	1	47	47	0

*Classification of active pesticide ingredient (IPCS, 2009).
n= 24 rats/ group. The pictures shaded mark the highest values.

Table 2. Signs of toxicity following insecticide application

4.2 Body weight

When body weights were analyzed, it was evident that rats exposed to CYP and DEL experienced delayed growth. The observed response was directly related to the insecticide concentration (Figure 2). The animals treated with MAL did not lose weight during the course of treatment were compared to the control group. A slight weight loss was observed in animals exposed to 4,4'-DDT. Animals treated with CHL showed a decrease in weight gain that was statistically significant *vs* control group. This effect is directly related to the insecticide concentration used in this study (Figure 2A).

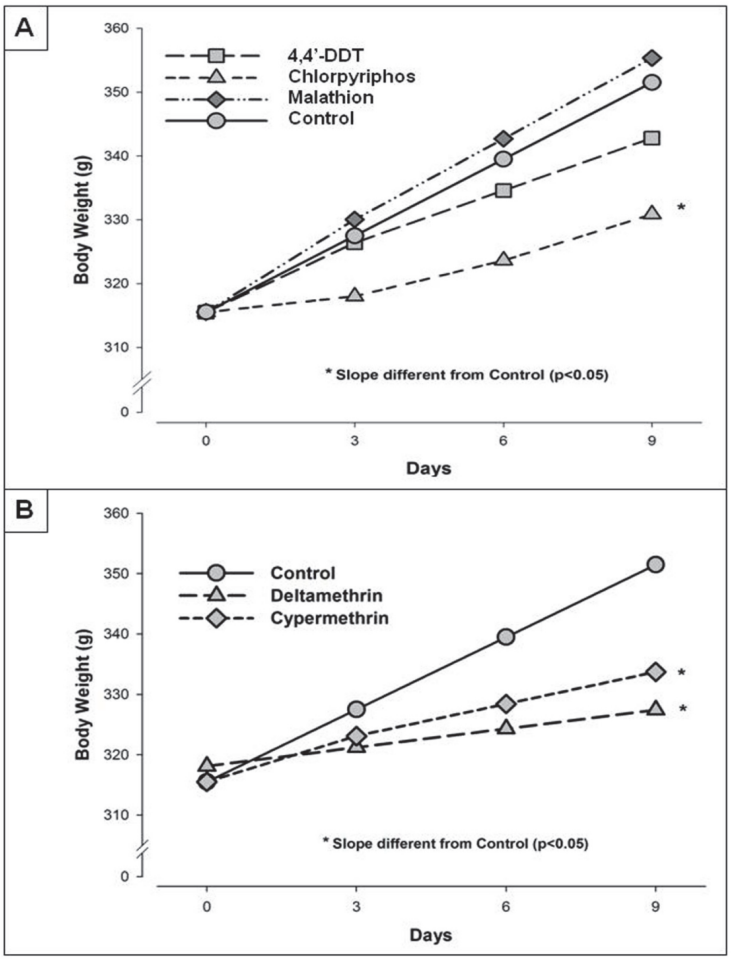


Fig. 2. Weight gain *vs* time.

4.3 Lipid profile

TG levels increased in all insecticide-treated groups after 6 day and these levels remained high until the end of treatment (Figure 3). This increase mainly reflects liver damage. However,

only the MAL-treated group showed decreased TG levels *vs* controls. Although the insecticide concentrations chosen for this study were based on levels detected in grains in previous work (Aldana et al., 2008), some of these concentrations MAL and CHL, DEL, CYP and 4,4'-DDT were within permissible limits in food for human consumption (Table 1). As shown in figure 3A, a cumulative effect was observed on day 9 of treatment, when all the insecticide-treated groups exhibited statistically significant increases in levels of TG ($p < 0.05$).

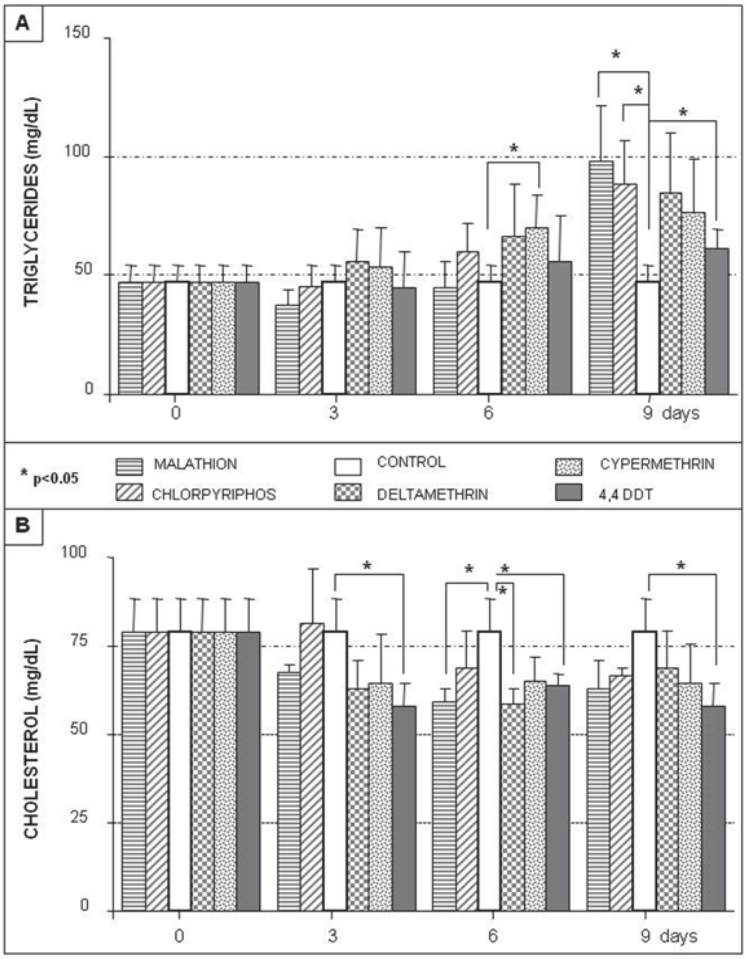


Fig. 3. Triglyceride and cholesterol levels. In (A), we show triglyceride (TG) levels during 0, 3, 6 and 9 days of consecutive insecticide treatment. Control group with (○), chlorpyriphos (●), 4,4'-DDT (Δ), deltamethrin (▲), malathion (□), cypermethrin (■). In (B), Cholesterol levels during 0, 3, 6 and 9 days of consecutive insecticide treatment. The different groups are represented by the same symbols used in (A).

Cholesterol levels typically decrease after exposure to insecticides (Aldana et al., 1998; Aldana et al., 2001). However, the reduction in the CHL-treated group was very small and was not observed until day 6 after treatment. The 4,4'-DDT-treated group exhibited the greatest reduction in cholesterol levels and the MAL-treated group showed the smallest decrease. CYP treatment showed a decrease in serum cholesterol on day 3 of treatment and that remained unchanged until the end of treatment (day 9) (Figure 3B). The decrease in serum cholesterol levels and the increase in TG levels confirm these insecticides caused liver damage.

4.4 Liver damage

In the case of the MAL- and CHL-treated groups, the expected decreased in albumin level was absent. On the contrary, albumin levels remained slightly elevated *vs* the control group (MAL, $p < 0.05$). Neither MAL nor CHL induced an acute phase response as evidenced by this marker.

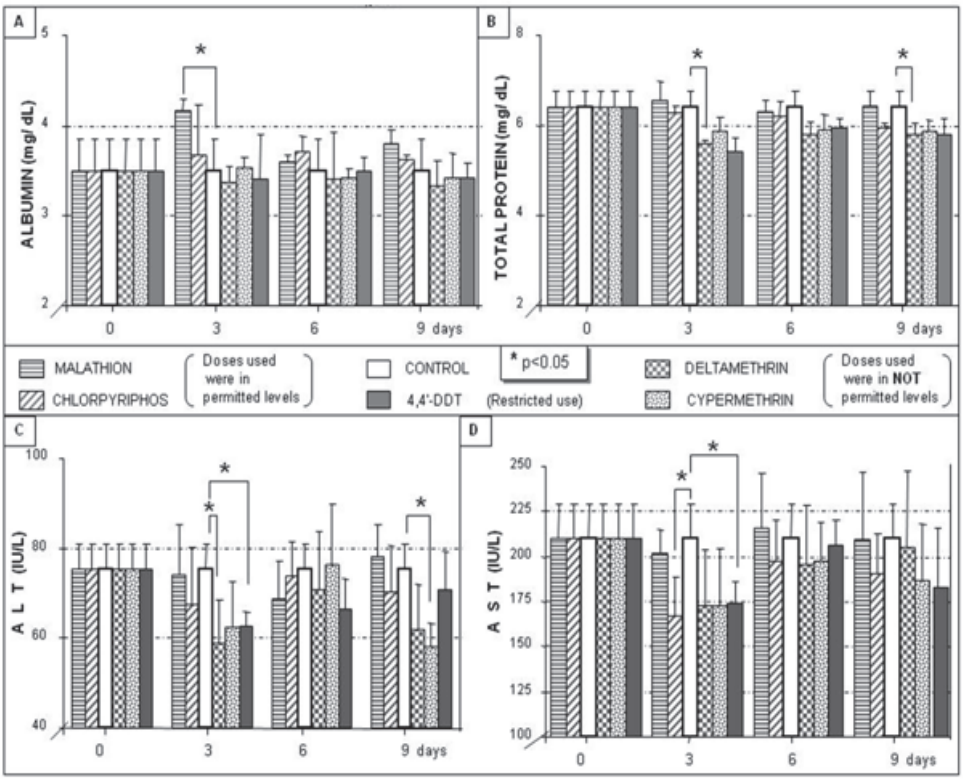


Fig. 4. Liver function tests. (A) Albumin levels were measured on days 0, 3, 6 and 9 of insecticide administration. Control is in open bars in the middle; insecticides whose used concentrations were within permitted limits are on its left side; insecticides used in concentrations higher than those permitted by the Mexican Official standard appear on its right side (B) Total protein concentration, (C) Alanine transferase (ALT) activity, and (D) Aspartate aminotransferase (AST).

Total protein levels, exhibited a similar pattern to albumin levels (Figure 4A). Only DEL, CYP, and 4,4′-DDT had decreased protein levels *vs* the control group (Figure 4B).
ALT and ASL are biomarkers of liver damage; that typically increase after insecticide administration. However, both enzymes showed an initial decrease followed by a return to normal levels on day 6 (Figure 4C, 4D). The MAL-treated group, showed minimal changes in enzyme levels *vs* to the control.

4.5 Electron microscopy studies

The results of electron microscopy included changes in liver structure following treatment that were much more pronounced after 9 days of insecticide treatment.
Figure 5A shows the control liver from an animal treated only with pluronic. The central vein is shown surrounded by cords of normal hepatocytes, along with some sinusoids that are occupied by erythrocytes. Figure 5B shows control untreated liver. The ultrastructure of a normal hepatocyte with cytoplasm containing abundant mitochondria, lysosomes and intact rough endoplasmic reticulum are also shown.
Figure 6 shows the ultrastructure of hepatocytes from animals treated with different insecticides. DEL shows lipid droplets of different sizes and mitochondria with varying degrees of damage (Figure 6A). MAL shows liver with abundant clear and irregular vesicles. Severe damage to mitochondria is observed (Figure 6B). CHL shows mitochondria with varying degrees of damage. Lipid droplets and areas with glycogen are also present (Figure 6C). 4,4′-DDT shows extensive cytoplasmic damage and multiple irregular vesicles and mitochondria (Figure 6D).

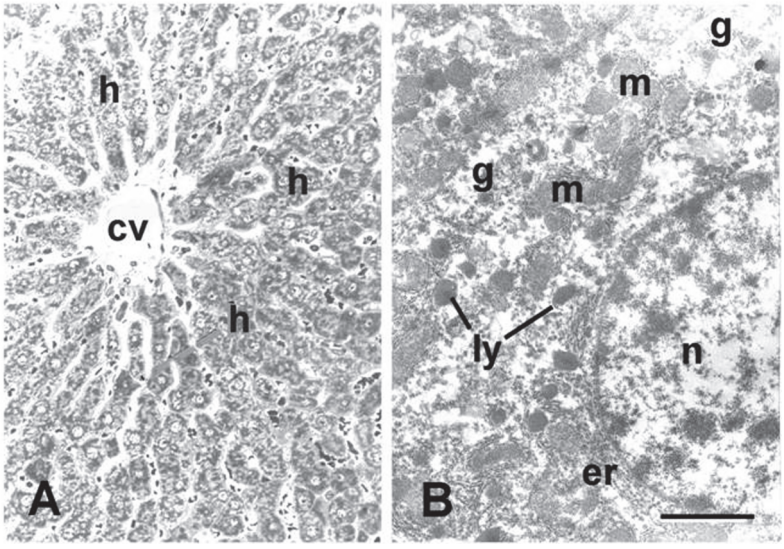


Fig. 5. Histopathology/Hepatocyte tissue sections. A). Control liver from animal treated only with pluronic. Central vein (cv) is shown surrounded by cords of normal hepatocytes (h). Some sinusoids are occupied by erythrocytes (x 20). B). Control untreated liver. Ultrastructure of a normal typical hepatocyte shows a round nucleus (n) and a cytoplasm with abundant mitochondria (m), lysosomes (ly), rough endoplasmic reticulum and clear areas with glycogen. Scale= 1.5 µm.

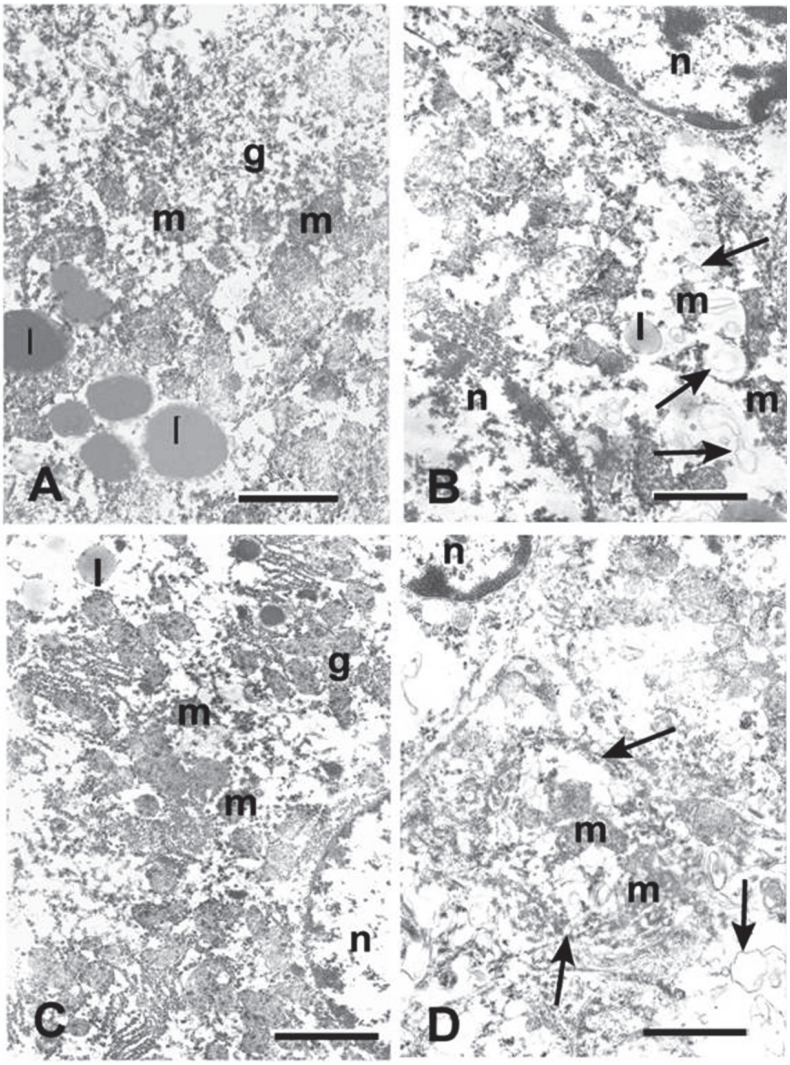


Fig. 6. Ultrastructure of hepatocytes from animals treated with different insecticides. A). Nine days after treated with deltamethrin. Lipid droplets (l) of different sizes, and mitochondria (m) with a different degree of damage are shown. Scarce rough endoplasmic reticulum and clear areas of glycogen (g) are observed. B). Animal treated 9 days with malathion. Abundant clear and irregular vesicles (arrows) are shown, along with severe damage of mitochondria (m). Lipid (l) droplet. Nuclei (n). C). Hepatocyte from animal treated with chlorpyriphos for 9 days. Similar changes were observed as in malathion treated animals. Mitochondria (m) with a different degree of damage, and lipid (l) droplets are observed. Areas with glycogen (g) are also shown. Nucleus (n). D). Animal treated with 4,4'-DDT. Extensive cytoplasmic damage is observed. Multiple irregular vesicles (arrows) and damaged mitochondria (m) are seen. Nucleus (n). Scale = 1.5 μm.

5. Discussion

To evaluate the hepatic effects of insecticides in rats, the doses used in the present study were similar to those found in stored grains in the state of Sonora. In some cases, the doses were within or above permissible limits. Still, it is clear that each insecticide affected the markers used, animal behavior, animal weight, appearance, alteration of lipid profile and liver function, and caused hepatocellular damage.

The results of this study indicate that CHL may have a different mechanism compared to CYP and DEL, even though they all belong to the moderately toxic group of compounds. In this context, it is important to consider both the toxicity and the concentration used for each insecticide (Table 1), as noted by other researchers (Manna et al., 2004).

Regarding the symptoms of shown by the animals after insecticide exposures, the results of the present research demonstrate that weight gain/loss measurement was a good qualitative parameter to evaluate toxicity as shown in the results obtained with MAL (Naraharisetti et al., 2008), with CHL (Meggs & Brewer, 2007), with pyrethroid CYP by our research group (Aldana et al., 2001; Aldana et al., 1998), and by others (Hussain et al., 2009).

The lipid profile also indicated the presence of liver damage, as evidenced by the decrease in cholesterol levels and the increase in TG levels after day 3 of treatment. In a recent study (Lasram et al., 2009) of acute toxicity at higher doses than used here, the same cholesterol pattern was observed with a transient level decrease, followed by a gradual return to normal levels. Plasma TG also increased significantly in their study. TG levels in our study increased progressively until the end of treatment, as has been observed during acute CYP intoxication reported in other studies at higher doses (Eraslan et al., 2008).

In relation to albumin, an acute phase protein, our results indicated that doses of CYP and DEL which were above permissible limits, caused a decrease albumin levels. On the other hand, when MAL and CHL were administered at a lower dose (within permissible limits), in albumin levels did not decrease.

In our study, the CHL-treated group behaved similar to the control group (Figure 4B). MAL caused slight increase in total protein values *vs* the control group.

Total protein served as a good biomarker of acute damage to liver because it partially reflects the decrease in albumin levels.

ALT and AST did not evoke immediate liver damage. Levels of both enzymes began to increase after day 3, indicating that these insecticides were provoking cumulative liver damage by inducing slight necrosis or cell damage.

Severe liver damage was not observed immediately after MAL exposure. The effect of MAL exposure were noticeable after day 6, suggesting a cumulative effect that is manifested more slowly than with other insecticides.

MAL gave rise to an acute phase response at the dose used in this study (within permissible limits), but it was the compound that elicited the lowest degree of liver damage.

Our results confirm others studies (Rezg et al., 2008) that observed an increase in AST and ALT following administration of MAL at 100 mg/kg for 32 days, while hepatic proteins and lipid content decreased significantly (Rezg et al., 2007).

These data show that serum albumin is the best biomarker to evaluate liver damage, since its levels remained low during the 9 days of treatment in relation to the concentration of insecticides. The liver damage observed in these studies was also confirmed with hiystopathological analysis, in which mitochondrial damage, presence of lipid droplets, and rough endoplasmic reticulum fragmentation were noticed as the most significant changes. The observed cell lysis was produced on the last days of treatment. We administered insecticides for 9 consecutive days and this sub-acute treatment more closely mimics human

exposure. Grains and grain products with insecticide residues at low doses are typically consumed over long periods of time. Our results are in agreement with another study in which MAL was administered at 33.051 mg/kg/day in Wistar rats via ip injection for 40 days (Saadi et al., 2008) and observed alterations with cytotoxicity signs in liver, lungs, bone marrow and testicles, caused by the high MAL concentrations.

The typical human exposure to pesticides is by oral consumption of grains. We chose the ip route of administration in this study, because it has a large surface of absorption and great vascularization, thus allowing for a rapid absorption. Also, these pesticides are very irritating to the animal's gastro intestinal tract when administered orally. Such so this irritation would have interfered with proper feeding.

6. Conclusion

The present study conducted with Wistar rats exposed to insecticides MAL, CHL, CYP, DEL and 4,4′ DDT at levels found in stored grains in Sonora, suggests possible hepatotoxic effects. These effects were manifested by biochemical alterations (decrease in serum cholesterol levels and increase in TG levels) as well as changes in the liver characterized by the presence of lipid droplets and fragmentation of rough endoplasmic reticulum. The most useful biomarker for evaluating hepatic damage was serum albumin. Although we cannot correlate the results observed in rats directly to oral consumption in humans, they suggest the possibility that these insecticides may not be as safe as other studies have previously reported. The insecticides examined in this study can provoke hepatic damage in rats and humans and these effects can be magnified under conditions of chronic exposure. Further studies are necessary to determine the effect of chronic exposure to these insecticides in rats and humans. A critical component of such studies should include monitoring of pesticide residue levels in grains and grain products.

7. References

Aldana, L., González de Mejía, E., Craigmill, A., Tsutsumi, V., Armendariz-Borunda, J., Panduro, A., & Rincón, AR. (1998). Cypermethrin increases apo A-1 and apo B mRNA but not hyperlipidemia in rats. *Toxicol Lett,* Vol.95, pp. 31-39, ISSN: 0378-4274

Aldana, L., Tsutsumi, V., Craigmill, A., Silveira, MI., & González de Mejía, E. (2001). α-Tocopherol modulates liver toxicity of the pyrethroid cypermethrin. *Toxicol Lett,* Vol.125, pp. 107-116, ISSN: 0378-4274

Aldana-Madrid, ML., Valdez-Hurtado, S., Vargas-Valdez, ND., Salazar-Lopez, NJ., Silveira-Gramont, MI., Loarca-Piña, FG., Rodriguez-Olibarria. G., Wong-Corral, FJ., Borboa-Flores, J., & Burgos-Hernández, A. (2008). Insecticide residues in stored grains in Sonora, Mexico: quantification and toxicity testing. *Bull Environ Contam Toxicol,* Vol.80, pp. 93-96, ISSN: 0007-4861

CICOPLAFEST. *Catálogo Oficial de Plaguicidas*. Comisión Intersecretarial para el Control del Proceso y Uso de Plaguicidas, Fertilizantes y Sustancias Tóxicas. (2004). México, D.F. Pp. 33, 40, 46, 96.

Eraslan, G., Kanbur, M., Silici, S., Altinordulu, S., & Karabacak, M. (2008). Effects of cypermethrin on some biochemical changes in rats: the protective role of propolis. *Exp Anim,* Vol.57, pp. 453-460, ISSN: 1341-1357

FAO/WHO. Food and Agriculture Organization of the United Nations. (1999). http://www.fao.org

Guillette, EA., Meza, MM., Aguilar, MG., Soto, AD., & García, IE. (1998). An anthropological approach to the evaluation of preschool children exposed to pesticides in Mexico. *Environ Health Perspect,* Vol.106, pp. 347-353, ISSN: 0091-6765

Holland, PT., Hamilton, D., Ohlin, B., & Skidmore, MW. (1994). Effects of storage and processing on pesticide residue in plant products. *Pure and Applied Chem,* Vol.66, pp. 335-356. ISSN: 0033-4545

Hussain, S., Khan, MZ., Khan, A., Javed, I., & Asi, MR. (2009). Toxico-pathological effects in rats induced by concurrent exposure to aflatoxin and cypermethrin. *Toxicon,* 53: 33-41, ISSN: 0041-0101

IFCC. International Foundation Chemical Clinical. (1986). BioMériux.

IPCS. International Program of the Chemical Safety. The WHO recommended classification of pesticides by hazard and guidelines to classification: (2009). Pp 65-78. WHO Press, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland. (NLM classification: WA 240) ISBN 978 92 4 154796 3, ISSN: 1684-1042

Lasram, MM., Annabi, AB., El Elj, N., Selmi, S., Kamoun, A., El-Fazaa, S., & Gharbi, N. (2009). Metabolic disorders of acute exposure to malathion in adult Wistar rats. *J Hazard Mater,* Vol.163, pp. 1052-1055, ISSN: 0304- 3894

Manna, S., Bhattacharyya, D., Mandal, TK., & Das, S. (2004).Repeated dose toxicity of alfa-cypermethrin in rats. *J Vet Sci,* Vol.5, pp. 41-45, ISSN: 1229-845X

Meggs, WJ. & Brewer, KL. (2007). Weight gain associated with chronic exposure to chlorpyrifos in rats. *J Med Toxicol,* Vol.3, pp. 89-93, ISSN: 1523-5130

Naraharisetti, SB., Aggarwal, M., Sarkar, SN., & Malik JK. (2008). Concurrent subacute exposure to arsenic through drinking water and malathion via diet in male rats: effects on hepatic drug-metabolizing enzymes. *Arch Toxicol,* Vol.82, pp. 543-551. ISSN: 0090-4341

Narayan, S., Dani, HM., & Misra, UK. (1990). Changes in lipid profiles of liver microsomes of rats following intratracheal administration of DDT or endosulfan. *J Environ Sci Health B,* Vol.25, pp. 243-257. ISSN: 0360-1234

Norma Oficial Mexicana (NOM-062-ZOO-1999). Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio. Modificated: november 26, 2008. Available at *www.senasica.gob.mx/default.asp?doc=743*

Pimentel Trevizan, LR., & Casadei de Baptista, G. (2000). Deltamethrin residues in wheat grains and their processed products evaluated by gas chromatography. *Scientia Agricola,* Vol. 57, pp. 199-203. ISSN 0188-4611

RAPAM, A.C. *Boletín de la Red de Acción sobre Plaguicidas y Alternativas en México.* (1997). No. 17, Enero-Abril.

Rezg, R., Mornagui, B., Kamoun, A., El-Fazaa, S., & Gharbi, N. (2007).Effect of subchronic exposure to malathion on metabolic parameters in the rat. *C R Biol,*Vol.330, pp. 143-147. ISSN 1768-3238

Rezg, R., Mornagui, B., El-Fazaa, S., & Gharbi, N. (2008). Biochemical evaluation of hepatic damage in subchronic exposure to malathion in rats: effect on superoxide dismutase and catalase activities using native PAGE. *C R Biol,* Vol.331, pp. 655-662. ISSN 1768-3238

Saadi, L., Lebaili, N., & Benyoussi, M. (2008). Exploration of cytotoxic effect of malathion on some rat organs structure. *Commun Agric Appl Biol Sci,* Vol.73, pp. 875-81. ISSN: 1379-1176

Shakoori, AR., Ali, RR., & Saleem, MA. (1998). Effects of six months' feeding of cypermethrin on the blood and liver of albino rats. *J Biochem Toxicol,* Vol.3, pp. 59-71. ISSN: 1095-6670

Videira, RA., Antunes-Madeira, MC., Lopes, VI., & Madeira, VM. (2001). Changes induced by malathion, methyl parathion and parathion on membrane lipid physicochemical properties correlate with their toxicity. *Biochim Biophys Acta,* Vol.1511, pp. 360-368. ISSN 0005-2736

Part 3

Analyses of Insecticides Action

13

Biochemical Analyses of Action of Chlorfluazuron as Reproductive Inhibitor in *Spodoptera litura*

Farzana Perveen
Chairperson, Department of Zoology
Hazara University, Garden Campus
Mansehra,
Pakistan

1. Introduction

Chlorfluazuron is highly effective against insect pests because it disrupts chitin synthesis during the moulting process (Retnakaran et al., 1989) and causes malformed larvae in *Spodoptera litura* (Omatsu et al., 1991). Chlorfluazuron had been used as chitin synthesis as well as reproductive inhibitor (Perveen, 2006). Toxicity and effects of chlorfluazuron on reproduction and viability of *S. litura* had been examined. The LD_{50} was found to be 12.0 ng larva^{-1} when evaluated up to pupation and 9.9 ng larva^{-1} up to adult emergence. Lethal dosages of chlorfluazuron when applied to newly-ecdysed fifth instars had a devastating effect on the *S. litura* population by killing them during larval, pupal, and adult stages. Reduction in the body weight was also observed in the larvae and pupae when treated with a sublethal dose (LD_{30}: 3.75 ng larva^{-1}) and in the adults when treated with sublethal doses (LD_{10}: 1.00 ng larva^{-1}; LD_{30}: 3.75 ng larva^{-1}) as newly-ecdysed fifth-instar larvae of *S. litura,* although the number of matings per female and life span of adult females and males remained unaffected by the same treatments. In insect pest management, the purpose of research is to maintain the pest population below a level of economic loss. Topical application of sublethal doses of chlorfluazuron (LD_{10} or LD_{30}) on newly-ecdysed fifth-instars did not kill the whole population of *S. litura* but reduced it, by affecting its reproduction. When sublethal doses were applied only to females or only to males, or both sexes, the results from these observations suggest that the fecundity was reduced to a similar degree when only females or only males or both sexes were treated with LD_{10} or LD_{30} doses as newly-ecdysed fifth-instars. However, the fertility and hatchability were affected more when only males were treated with LD^{10} and much more when treated with LD_{30} (Perveen, 2000a). However, there were no significant differences observed between larval and pupal treatment in the reduction of these biological parameters (Perveen, 2009a). Effects of chlorfluazuron on ovarian development and oögenesis (Perveen and Miyata, 2000), testicular development and spermatogenesis (Perveen, 2000b), insemination and number of inseminated sperm (Perveen, 2008) and haemolymph-borne oviposition-stimulating factors (Perveen, 2009b) in *S. litura* had been reported. The effect of sublethal doses of chlorfluazuron on embryogenesis of *S. litura* has also been reported during the

eight embryonic developmental stages (0-84 hours after oviposition) (Perveen, 2009c). Chlorfluazuron has proved significantly affected to the biological and reproductive parameters of *S. litura*. The biochemical analyses of effects of sublethal doses of chlorfluazuron and their efficacy on reproductive system of *S. litura* have been undertaken during the present research work. That research work will help in the development of a new group of pesticides that may be cheaper and less hazardous to the environment and non-target organisms which is the main desired outcome of the present work.

2. Experimental procedures

2.1 Insect rearing

Experiments were conducted with *Spodoptera litura* (F.) (Lepidoptera: Noctuidae) taken from a stock that was established from eggs obtained from Aburahi Laboratory of Shionogi Pharmaceutical (Koga-Shiga-Pref., Japan). The larvae of *S. litura* were reared in the laboratory under controlled conditions on the artificial diet Insecta LF® (Nihon Nohsan-kohgyo, Kanagawa, Japan). The rearing temperature was maintained at 25±1 °C, with a L16:D8 hour photoperiod and 50-60% r.h. To facilitate observations, the dark period was set from 06:00 to 14:00 hours. Adults were fed on a 10% sucrose solution soaked in cotton. The eggs, which were laid on Rido® cooking paper (Lion, Tokyo, Japan), were collected every 3rd day and kept in 90 ml plastic cups (4 cm in diameter4×4 cm high) for hatching under the same environmental conditions (Perveen, 2000a).

2.2 Chlorfluazuron and its application

Chlorfluazuron (Atabron®) in powder form at 99.9% purity was obtained from Ishihara Sangyo Kaisha and was stored at 4 °C until use. It was diluted with acetone (analytical grade; Wako Pure Chemical Industries, Tokyo, Japan) for the test concentrations (Perveen, 2000a).

Using a micro-applicator (Kiya, Tokyo, Japan), sublethal doses (LD_{10}: 1.00 ng larva^{-1} or LD_{30}: 3.75 ng larva^{-1}) of chlorfluazuron diluted in 2.0 µl of acetone were applied topically using a micro-syringe (Ito, Fuji-City, Japan) to the dorsum of the third or fourth thoracic segments of newly-ecdysed fifth-instars of *S. litura* of similar weight (approximately 131 mg) and size (1.6 cm long). The LD_{10} or LD_{30} values were calculated based on the results of the toxicity data of larval tests at adult emergence (Perveen, 2000a). A batch of larvae treated with 2.0 µl of acetone was kept as a control to determine any effects of the solvent. Treated, untreated, and control batches of larvae were kept in paper towel-padded 860-ml plastic cups (13 cm in diameter×9.5 cm high; n=150 for each batch) and provided with food on alternate days. These batches were kept under the same environmental conditions as those used for the rearing stock culture. Treated, untreated, and control larvae were examined daily and mortality was recorded until adult emergence. The mortality was observed in the control larvae were the same as in the untreated ones. Therefore, their data were not used for further analysis. Pupae were sexed immediately following pupation. The larvae, pupae and adults taken from treated and untreated batches were use separately for analysis of effects of chlorfluazuron on ovarian, testicular and egg constituents. When the male and female adults were emerged, they were used for the experiments in the sections described below (Perveen, 2000a).

2.3 Quantitative determination of ovarian constituents

2.3.1 Estimation of ovarian protein, carbohydrate and lipid

Paired ovaries were collected from newly emerged adults in the untreated and treated (LD_{10} or LD_{30}) batches (n=15–21) and analysed individually as follows:

Protein was extracted according to Le Bras and Echaubard (1977). The ovaries were homogenised in aqueous trichloroacetic acid (TCA: 1.0 ml; 100 g l^{-1}) and centrifuged at 1844 g for 10 min at 4 °C. The supernatant (1) was used for carbohydrate determination while the precipitate was washed with ether and chloroform (1.0 ml; 1: 1 by volume) to remove lipids. After centrifugation as before, the chloroform supernatant (2) was used for lipid determination and the second precipitate, thus obtained, was suspended in distilled water (1.0 ml) and the amount of protein was determined in an aliquot using the Coomassie blue method (Bradford, 1976) at 595 nm with bovine serum albumin (Sigma Chemical Corp., Tokyo, Japan) as a standard.

Carbohydrate content was determined by the anthrone method (Duchateau and Florkin, 1959; Mokrasch, 1954) as used previously for haemolymph carbohydrate determination (Soltani, 1990). After ovarian extraction by TCA and centrifugation as before, an aliquot was taken from the supernatant (1) for carbohydrate quantification at 625 nm using trehalose (Merck Chemical Corp., Tokyo, Japan) as a standard.

Lipids were measured according to Goldsworthy et al. (1972). After lipid extraction an aliquot (0.1 ml) from supernatant (2) was mixed with concentrated sulphuric acid (1.0 ml) and heated for 10 min at 100°C. After cooling, an aliquot was taken and mixed with a solution of 13 mM vanillin in 11.8 M phosphoric acid (Wako Pure Chemical Industries Ltd., Tokyo, Japan). Absorbance was measured at 545 nm in a microplate reader with a computer, using a standard lipid solution of cholesterol (Wako Pure Chemical Industries Ltd., Tokyo, Japan) containing 1.0 g 100 ml^{-1} as a standard.

Data on ovarian constituents were expressed as µg pair of $ovaries^{-1}$ and µg mg of $ovaries^{-1}$ (Perveen and Miyata, 2000).

2.3.2 Estimation of ovarian nucleic acids

Estimation of nucleic acids (RNA and DNA) in the ovaries was determined separately at three different stages of development of *S. litura,* which were as follows: (1) newly ecdysed last (sixth)-instars; (2) newly-pupated pupae and (3) newly-emerged female adults. Paired ovaries were collected from the relevant stages of the untreated and treated (LD_{10} or LD_{30}) batches (n=10) and weighed 81.1 mg and 10% homogenate was prepared in distilled water. DNA and RNA were extracted according to the procedure of Schmidt and Thannhauser (1945) with some modified according Munro (1966). DNA and RNA were measured by the diphenylamine method of Burton (1956) and orcinol reaction of Schneider (1957), respectively (Perveen and Miyata, 2000).

2.3.2.1 Extraction of DNA and RNA

Ovaries from 4–8 insects ca. 14–28 mg were used for each batch (untreated and treated). Ovaries were crushed in 2.0 ml 70% ethanol and centrifuged at 1180 g for 15 min. The supernatant was discarded and 2.0 ml of 70% ethanol was added in the precipitate and centrifuged at same speed. This process of ethanol washing was repeated three times. The supernatant was discarded and 2.0 ml methanol and ether (3:1 ratio) was added and boiled for 3 min, the solution was cooled and centrifuged for 15 min at 1180 g. The supernatant was discarded and the precipitate was treated twice with the ethanol and ether mixture (1:1). The

supernatant was discarded and the (1:1) was desiccated in a vacuum under the pressure of KOH (pellets) for 2 hours and then 2.0 ml of ice-cold water were added to the precipitate and Allowed to dissolve and was kept in refrigerator for 1 hour. Two ml 20% ice-cold PCA was added. The mixture was incubated at 4 °C in the refrigerator for 18–24 hours. The product contained RNA and precipitate contained DNA. Two ml of 10% PCA was added, mixed, shaken well and heated at 75 °C for 45 min. The supernatant represented the DNA extracted (Perveen, accepted).

2.3.2.2 Estimation of ovarian DNA and RNA

A DNA extract (0.4 ml) was taken for the test and 0.4 ml 10% PCA for the blank. Distilled water 0.6 ml and then 2.0 ml diphenylamine reagent were added to each tube. They were mixed and boiled for 15 min. Blue colour indicated the presence of DNA. Absorbance was read 660 nm against a blank. The amount of DNA of the ovaries was calculated by the following formula (Perveen, accepted):

$$\mu g \text{ DNA mg ovary}^{-1} = \frac{\text{total } \mu g \text{ from curve} \times \text{dilution}}{1 \times \textit{weight of ovaries} \times 0.1 \times 1000}$$

An RNA extract of 0.2 ml was taken for each test in a separate test and 1.8 ml distilled water added to it and 2.0 ml was added to the blank. Then 2.0 ml of orcinol reagent was added to the tests and the blank. These were mixed well and boiled for about half an hour. When green colour appeared, the absorbance was read at 660 nm against the blanks. The amount of RNA of ovaries was calculated by the following formula (Perveen, accepted):

$$\mu g \text{ RNA mg ovary}^{-1} = \frac{\text{total } \mu g \text{ from curve} \times \text{dilution}}{2 \times \textit{weight of ovaries} \times 0.1 \times 1000}$$

2.3.2.3 Estimation of utricular DNA of spermatheca

The *utriculus* is that part of spermatheca in female in which the sperm are stored after mating. Therefore, estimation of the nucleic acids were determined in the *utriculi* which were collected after first and second mating of the untreated and treated (LD_{10} or LD_{30}) batches (n=5) and weighed and a 10% homogenate was prepared in distilled water. The rest of the procedure was the same as described above in section 1.2.2 (Perveen, accepted).

2.3.3 Estimation of ovarian ecdysteroid titre

Analysis of the ecdysteroid titre in ovaries was determined in 7 day-old female pupae to 4 day-old female adults after each consecutive 24 hours. Ovaries of the untreated and treated batches (n=5–14) were used. Ovaries were dissected in 0.9% NaCl solution and incubated in Ringer's solution (Barbosa, 1974) for 8 hours. Five pairs of ovaries with 300 µl 70% methanol were homogenized at 10625 g for 10 min. The supernatant obtained was added to the previous one and 500 µl of hexane was added and centrifuged at 1844 g for 5 min. The supernatant was dried under nitrogen gas (N_2) and 1.0 ml 5% methanol was added. This solution was passed through a C_{18} sep-pak (Millipore) for fractionation (Rees and Issac, 1985). Then 1.0 ml 70% methanol was added and this solution was dried by N_2 gas (Perveen, accepted).

2.3.3.1 RIA methods

These samples were tested by the method described by Horn et al. (1976) and Gilbert et al. (1980). However, the ecdysteroid titre, antiecdysteroid antiserum was obtained from Prof.

Dr. LI Gilbert and Prof. Dr. WE Bollenbacher (University of North Carolina). The [^{3}H] ecdysone (1.85 TBq mmol^{-1}) was obtained from Du Pont Company Ltd., North Carolina, USA and 20-hydroxyecdysone from Rohto Pharmaceutical Company, New York, USA (Perveen, accepted).

Samples were dried and added to 50 µl distilled water. The standard groups (2–3 replicates), 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0 and 0.2 ng 20-hydroxyecdysone 50 µl^{-1} were made. The [^{3}H] ecdysone solution (9.8 ml distilled water and 200 µl ^{3}H] was made and added to 50 µl of [^{3}Hecdysone 0.01 µci sample^{-1}) was vortexed. Then, 50 µl antiserum (appropriate dilution of the antiserum) was added and vortexed. It incubated at 5 °C for overnight. Then 150 µl saturated solution of ammonium sulphate was added. It was again incubated on ice for 0.5–1 hour and centrifuged at 1180 g for 5 min. The supernatant was removed by aspiration and 300 µl 50% sat. ammonium sulphate was added and vortexed. Again the tubes were centrifuged at 1180 g for 5 min. The supernatant was removed by aspiration and 20 µl distilled water was added and vortexed. The 300 µl of scintillator was added and vortexed. Tubes were then radio-assayed (Perveen, accepted).

2.4 Quantitative determination of testicular constituents

2.4.1 Estimation of testicular protein, carbohydrate and lipid

Testes were dissected from newly emerged adults in the untreated and treated (LD_{10} or LD_{30}) batches (n=11–15), and analysed individually by the same procedure as described above in section 1.2.1. Data were expressed as µg of testicular protein, carbohydrate and lipids in mg of testis^{-1} and µg testis^{-1} (Perveen and Miyata, 2000).

2.4.2 Estimation of testicular nucleic acids

Analysis of nucleic acids (RNA and DNA) in testes was determined separately at the different stages of development of *S. litura,* i.e., larvae, pupae and adults. The testes were dissected from the relevant stage of untreated and treated (LD_{10}: 1.00 ng larva^{-1} or LD_{30}: 3.75 ng larva^{-1}) batches (n=5) in Ringer' solution. Testes from 4–8 moths were pooled, about 14–28 mg weighed then a 10% homogenate was prepared in distilled water and the further procedure used was the same as described above in section 1.2.2 (Perveen, accepted).

2.4.3 Estimation of seminal vesicular DNA

Seminal vesicles (paired) are wide tube like structure arising from the testis. During mating, mature sperm transferred from the seminal vesicles through vas efferent and packed into spermatophore. Here, sperm are stored for some time. For estimation of DNA in seminal vesicles, they were collected from individuals, before adult emergence, newly emerged adults and 1 day-old adults of untreated and treated (LD_{10} or LD_{30}) batches (n=5) and weighed. A 10% homogenate was prepared in distilled water. The further procedure was the same as described above in section 1.2.2 (Perveen, accepted).

2.4.4 Estimation of aedeagular DNA

The *Aedeagus* is a 1.0 cm tube, present in the area of collum (part of spermatophore) formation of the cuticular secondary segment of the ductus ejaculatorius simplex. For the estimation of DNA, it was collected from individuals, before adult emergence, newly adult emergence and 1 day-old adults of the untreated and treated (LD_{10}: 1.00 ng larva^{-1} or LD_{30}: 3.75 ng larva^{-1}) batches (n=5) and weighed. A 10% homogenate was prepared in distilled

water. The further procedure was the same as described above in section 1.2.2 (Perveen, accepted).

2.4.5 Estimation of testicular ecdysteroid titre

Analysis of the ecdysteroid titre in testes was determined in newly-ecdysed sixth-instars to pre-pupae (72 hours) after each consecutive 8 hours. Testes of the untreated and treated batches (n=5) were used. Testes were dissected in 0.9% NaCl solution and incubated in Ringer's solution for 8 hours. Five pairs of testes with 300 µl 70% methanol were homogenized at 10625 g for 10 min. Then the same procedure was used as described above in section 1.2.3 (Perveen, accepted).

2.5 Data analysis

Data were analyzed by using analysis of variance (ANOVA) (Concepts, 1989; Minitab, 1997; Walpol and Myers, 1998) at $P<0.0001$ and Scheffe's F-test (multiple range) (Scheffe, 1953) at 5%.

3. Results

3.1 Effects on ovarian constituents

3.1.1 Effects on ovarian protein

Quantitative determination of the protein content in each pair of ovaries of newly emerged female adults showed that sublethal doses of chlorfluazuron significantly reduced the amount of protein in the ovaries (calculated in µg protein mg ovary^{-1} or µg protein pair of ovaries^{-1}: $P<0.0001$) compared with the controls, but there was no significant difference ($P=0.6385$) between the LD_{10} and LD_{30} treatments (Table 3.1). The protein content in the control newly emerged female adults was 0.71±0.18 µg mg ovary^{-1} or 53.9±18.5 µg pair ovaries^{-1}. However, in the LD_{10}-treated, newly emerged female adults, it was 0.45±0.12 µg mg ovary^{-1} or 28.8±13.1 µg pair ovaries^{-1}. In the LD_{30}-treated, newly emerged female adults, it was 0.42±0.10 µg mg ovary^{-1} or 24.5±10.6 µg pair of ovaries^{-1} (Table 3.1) (Perveen and Miyata, 2000).

Treatments[1]	n	Ovarian protein content[2]	
		(M±SD) µg pair of ovaries^{-1}	(M±SD) µg mg of ovaries^{-1}
Control	15	53.9±18.5^{a}	0.71±0.18^{a}
LD_{10}	21	28.8±13.1^{b}	0.45±0.12^{b}
LD_{30}	17	24.5±10.6^{b}	0.42±0.10^{b}

[1]Source: Perveen and Miyata, 2000; LD_{10}, 1.00 ng larva^{-1}; LD_{30}, 3.75 ng larva^{-1}; n: number of females used.
[2]Data were analyzed by one-way ANOVA (Concepts, 1989) and Scheffe`s F-test (Scheffe, 1953) at 5%. Means within a column followed by different letters are significantly different ($P<0.0001$).

Table 3.1. Effect of sublethal doses of chlorfluazuron on the ovarian protein contents in newly-emerged adult female after topical application to newly-ecdysed fifth-instars of *Spodoptera litura* (Source: Perveen and Miyata, 2000).

3.1.2 Effects on ovarian carbohydrate

The carbohydrate content of ovaries was reduced, but the reduction was not significant ($P<0.0963$ and $P<0.0611$, respectively, when calculated for µg mg ovary^{-1} or µg pair of ovaries^{-1}) for the LD_{10} or LD_{30} treatments compared with controls (Table 3.2). In control, newly emerged female adults, the amount of carbohydrate estimated was 1.16±0.51 µg mg ovary^{-1} or 86.2±36.9 µg pair of ovaries^{-1}. However, in the LD_{10}-treated newly-emerged female adults, it was reduced by 15.5%, when considered in mg ovary or 24.5%, when considered per pair of ovaries. In the LD_{30}-treated newly-emerged female adults, it was reduced by 24%, when considered in mg ovary or 38.1%, when considered per pair of ovaries (Table 3.2) (Perveen and Miyata, 2000).

Treatments[1]	n	Ovarian carbohydrate content[2]	
		(M±SD) µg pair of ovaries^{-1}	(M±SD) µg mg of ovaries^{-1}
Control	15	86.2±36.9[a]	1.16±0.51[a]
LD_{10}	21	65.1±47.3[a]	0.98±0.55[a]
LD_{30}	17	53.4±39.5[a]	0.88±0.46[a]

[1]LD_{10}, 1.00 ng larva^{-1}; LD_{30}, 3.75 ng larva^{-1}; n: number of females used.
[2]Data were analyzed by one-way ANOVA (Concepts, 1989) and Scheffe`s F-test (Scheffe, 1953) at 5%. Means within a column followed by different letters are significantly different ($P<0.0001$).

Table 3.2. Effect of sublethal doses of chlorfluazuron on the ovarian carbohydrate contents in newly-emerged adult female after topical application to newly-ecdysed fifth-instars of *Spodoptera litura* (Source: Perveen and Miyata, 2000)

3.1.3 Effects on ovarian lipid

The lipid content of ovaries was reduced, but the reduction was not significant ($P<0.0963$ and $P<0.0611$, respectively, when calculated for µg mg ovary^{-1} and µg pair ovaries^{-1}) by the LD_{10} or LD_{30} treatments compared with the controls (Table 3.3). In control, newly-emerged female adults, the amount of lipid estimated was 8.49±2.23 µg mg^{-1} or 643.6±199.1 µg pair ovaries^{-1}. However, in the LD_{10}-treated newly-emerged female adults, it was reduced by 10%, when considered in mg of ovaries or 22.6%, when considered per pair of ovaries. In the LD_{30}-treated newly-emerged female adults, it was reduced by 16%, when considered in mg of ovaries or 32.2%, when considered per pair of ovaries (Table 3.3) (Perveen and Miyata, 2000).

Treatments[1]	n	Ovarian lipid content[2]	
		(M±SD) µg pair of ovaries^{-1}	(M±SD) µg mg of ovaries^{-1}
Control	15	643.6±199.1[a]	8.49±2.23[a]
LD_{10}	21	498.4±274.2[a]	7.64±3.08[a]
LD_{30}	17	436.6±245.2[a]	7.14±2.71[a]

[1]LD_{10}, 1.00 ng larva^{-1}; LD_{30}, 3.75 ng larva^{-1}; n: number of females used.
[2]Data were analyzed by one-way ANOVA (Concepts, 1989) and Scheffe`s F-test (Scheffe, 1953) at 5%. Means within a column followed by different letters are significantly different ($P<0.0001$).

Table 3.3. Effect of sublethal doses of chlorfluazuron on the ovarian lipid contents in newly-emerged adult female after topical application to newly-ecdysed fifth-instars of *Spodoptera litura* (Source: Perveen and Miyata, 2000)

3.1.4 Effects on ovarian DNA

Quantitative determination of the DNA of the ovaries of newly-ecdysed sixth-instar larvae, pupae and adults showed that the concentration of DNA was greater in larvae, and then it decreased again in pupae and then increased in female adults. Sublethal doses of chlorfluazuron significantly ($P<0.001$) reduced the amount of DNA in the LD_{10}-treated and more significantly ($P<0.0001$) reduced in the LD_{30}-treated females compared with the controls, measured either in µg mg of ovary^{-1} or µg pair of ovaries^{-1} at each developmental stage (newly-ecdysed last-(sixth)-instar larvae; newly ecdysed pupae; newly-emerged female adults) (Table 3.4) (Perveen, accepted).

In the controls newly-ecdysed last-(sixth)-instar larvae, the amount of DNA estimated was 4.74±0.94 µg mg^{-1} ovary or 10.03±0.56 µg pair ovaries^{-1}. However, in the LD_{10}-treated larvae, it was reduced to 2.67±0.62 µg mg ovary^{-1} or 8.19±0.90 µg pair ovaries^{-1}. In the LD_{30}-treated newly-emerged female adults, it was reduced to 1.27±0.43 µg mg ovary^{-1} or 6.66±0.53 µg pair ovaries^{-1} (Table 3.4) (Perveen, accepted).

In the control newly-pupated female pupae, the amount of DNA estimated was 3.00±0.62 µg mg ovary^{-1} or 17.91±0.61 µg pair ovaries^{-1}. In the LD_{10}-treated newly-pupated female pupae, it was 1.51±0.75 µg mg^{-1} ovary or 16.18±0.61 µg pair ovaries^{-1}. In the LD_{30}-treated newly emerged female adults, it was 0.50±0.07 µg mg ovaries^{-1} or 24.61±0.92 µg pair ovaries^{-1} (Table 3.4) (Perveen, accepted).

In the control newly emerged female adults, the amount of DNA estimated was 5.98±0.61 µg mg ovary^{-1} or 24.61±0.92 µg pair ovaries^{-1}. In the LD_{10}-treated newly-pupated female pupae, it was 4.81±0.54 µg mg ovary^{-1} or 22.95±0.57 µg pair ovaries^{-1}. In the LD_{30}-treated newly-emerged female adults, it was 3.50±0.62 µg mg ovary^{-1} or 21.19±0.91 µg pair ovaries^{-1} (Table 3.4) (Perveen, accepted).

Treatment stages	Treat-ments[1]	n	Ovarian DNA contents[2]	
			(M±SD) µg mg^{-1}	(M±SD) µg pair ovaries^{-1}
Newly ecdysed larvae	Control	10	4.74±0.94^{a}	10.03±0.56^{a}
	LD_{10}	10	2.67±0.62^{b}	8.19±0.90^{b}
	LD_{30}	10	1.27±0.43^{c}	6.66±0.53^{c}
Newly pupated pupae	Control	10	3.00±0.62^{a}	17.91±0.61^{a}
	LD_{10}	10	1.51±0.75^{b}	16.18±0.61^{b}
	LD_{30}	10	0.50±0.07^{c}	14.7±0.62^{c}
Newly emerged adults	Control	10	5.98±0.61^{a}	24.61±0.92^{a}
	LD_{10}	10	4.81±0.54^{b}	22.95±0.57^{b}
	LD_{30}	10	3.50±0.62^{c}	21.19±0.91^{c}

[1]LD_{10}, 1.00 ng larva^{-1}; LD_{30}, 3.75 ng larva^{-1}; n: number of females used.
[2]Data were analyzed by one-way ANOVA (Concepts, 1989) and Scheffe`s F-test (Scheffe, 1953) at 5%. Means within a column followed by different letters are significantly different ($P<0.0001$).

Table 3.4. Effects of sublethal doses of chlorfluazuron on the ovarian DNA contents of different developmental stages after topical application to newly-ecdysed fifth-instars of *Spodoptera litura* (Source: Perveen, accepted).

3.1.5 Effects on utricular DNA in spermatheca

Quantitative estimation of the DNA of the utriculus of the spermatheca after first and second matings showed that it was greater after the first mating than the second mating

(Table 3.6). Sublethal doses of chlorfluazuron significantly (P<0. 001) lowered the amount of DNA in the utriculus of the spermatheca in the LD_{10}- and more significantly (P<0.0001) reduced in the LD_{30}-treated females compared with the controls, as measured in µg mg tissue^{-1} (Table 3.5) (Perveen, accepted).
In the control, after the first mating, the amount of DNA estimated in the utriculus of the spermatheca was 2.04±0.06 µg mg^{-1}. In the LD_{10}- treated larvae, it was reduced by 38.7%. In the LD_{30}-treated larvae, it was reduced by 58.3% (Table 3.5) (Perveen, accepted).
In the control after the second mating, the amount of DNA estimated in the utriculus of the spermatheca was 1.75±0.08 µg mg^{-1}. In the LD_{10}- treated larvae, it was reduced by 32.0%. In the LD_{30}-treated larvae, it was reduced by 57.1% (Table 3.5) (Perveen, accepted).

Treatments[1]	n	After first mating[b] (M±SD) µg mg^{-1}	After second mating[2] (M±SD) µg mg^{-1}
Control	5	2.04±0.06[a]	1.75±0.08[a]
LD_{10}	5	1.25±0.09[b]	1.19±0.07[b]
LD_{30}	5	0.85±0.08[c]	0.75±0.08[c]

[1]LD_{10}, 1.00 ng larva^{-1}; LD_{30}, 3.75 ng larva^{-1}; n: number of females used.
[2]Data were analyzed by one-way ANOVA (Concepts, 1989) and Scheffe`s F-test (Scheffe, 1953) at 5%. Means within a column followed by different letters are significantly different (P<0.0001).

Table 3.5. Effects of sublethal doses of chlorfluazuron after the 1st and 2nd matings on the DNA contents of the *utriculus* of the spermatheca of adult females after topical application to newly-ecdysed fifth-instars of *Spodoptera litura* (Source: Perveen, accepted).

3.1.6 Effects on ovarian RNA

Quantitative determination of the RNA of the ovaries of newly-ecdysed sixth instar larvae, pupae and female adults showed that the concentration of RNA was the greatest in larvae then it decreased in pupae and then increased again in adult females as for DNA estimation in female ovaries (Table 3.6). Sublethal doses of chlorfluazuron significantly (P<0.001) lowered the amount of RNA in the LD_{10}-and more significantly (P<0.0001) lowered the RNA in the LD_{30}-treated females compared with the controls, measured in µg mg ovary^{-1} or µg pair ovaries^{-1} in each developmental stage in (newly-ecdysed sixth-instar larvae; newly-pupated pupae; newly-emerged male adults (Table 3.6) (Perveen, accepted).
In the control newly-ecdysed sixth-instar larvae, the amount of RNA estimated was 51.91±0.50 µg mg ovary^{-1} or 242.90±11.69 µg pair ovaries^{-1}. In the LD_{10}-treated larvae, it was 47.59±1.09 µg mg ovary^{-1} or 220.64±6.76 µg pair ovaries^{-1}. In the LD_{30}-treated newly-emerged female adults, it was 43.29±0.82 µg mg ovary^{-1} or 206.33±5.33 µg pair ovaries^{-1} (Table 3.5). In the control newly-pupated female pupae, the amount of RNA estimated was 25.70±0.47 µg mg ovary^{-1} or 220.07±6.15 µg pair ovaries^{-1}. In the LD_{10}-treated newly-pupated female pupae, it was 24.25±0.84 µg mg ovary^{-1} or 209.13±6.19 µg pair ovaries^{-1}. In the LD_{30}-treated newly emerged female adults, it was 22.19±0.75 µg mg ovary^{-1} or 199.45±4.09 µg pair ovaries^{-1} (Table 3.6) (Perveen, accepted).
In the control newly-emerged female adults, the amount of RNA estimated was 50.12±0.63 µg mg ovary^{-1} or 235.79±9.06 µg pair ovaries^{-1}. In the LD_{10}-treated newly-emerged female adults, it was 48.75±0.62 µg mg ovary^{-1} or 219.94±4.45 µg pair ovaries^{-1}. In the LD_{30}-treated newly-emerged female adults, it was 47.54±0.62 µg mg ovary^{-1} or 241.51±3.61 µg pair ovaries^{-1} (Table 3.6) (Perveen, accepted).

Treatment stages[1]	Treat-ments[1]	n	Ovarian RNA contents[2]	
			(M±SD) µg mg^{-1}	(M±SD) µg pair ovaries^{-1}
Newly ecdysed larvae	Control	10	51.91±0.50^{a}	242.90±11.69^{a}
	LD_{10}	10	47.59±1.09^{b}	220.64±6.76^{b}
	LD_{30}	10	43.29±0.82^{c}	206.33±5.33^{c}
Newly pupated pupae	Control	10	25.70±0.47^{a}	220.07±6.15^{a}
	LD_{10}	10	24.25±0.84^{b}	209.13±6.19^{b}
	LD_{30}	10	22.19±0.75^{c}	199.45±4.09^{c}
Newly emerged adults	Control	10	50.12±0.63^{a}	235.79±9.06^{a}
	LD_{10}	10	48.75±0.62^{b}	219.94±4.45^{b}
	LD_{30}	10	47.54±0.62^{c}	214.51±3.61^{c}

[1]LD_{10}, 1.00 ng larva^{-1}; LD_{30}, 3.75 ng larva^{-1}; n: number of females used.
[2]Data were analyzed by one-way ANOVA (Concepts, 1989) and Scheffe`s F-test (Scheffe, 1953) at 5%. Means within a column followed by different letters are significantly different (P<0.0001).

Table 3.6. Effects of sublethal doses of chlorfluazuron on the ovarian RNA contents of different developmental stages after topical application to newly-ecdysed fifth-instars of *Spodoptera litura* (Perveen, accepted).

3.1.7 Effects on ovarian ecdysteroid titre

Ecdysteroid titre in ovaries was determined after each consecutive 24 hours on the 7 day-old female pupae through 4 day-old adult females. Preliminary data from controls indicated that the ovarian amounts of ecdysteroids, measured *in vivo*, changed during vitellogenesis in *S. litura* in a characteristic way: the amounts were low during pre-vitellogenesis (on the –4th day: 3.5±0.8 pg mg^{-1}; on the –3rd day: 4.5±0.5 pg mg^{-1}, after adult emergence), increased during vitellogenesis (on the –2nd day: 18.0±3.16 pg mg^{-1}; on the –1st day: 27.0±3.3 pg mg^{-1}; on the 0 day after adult emergence: 35.0±1.6 pg $^{-1}$mg, after adult emergence), peaked at choriogenesis (on the 1st day after adult emergence: 52.0±1.5 pg mg^{-1}) and decreased when the insects started to deposit eggs (on the 2nd day: 15.0±1.58 pg mg^{-1}) and decreased thereafter (Fig. 3.1).

Chlorfluazuron at the two tested sublethal doses significantly affected the amounts of ecdysteroids accumulated *in vivo* by ovaries. It significantly (P<0.001) decreased the amount in the LD_{10}-treated females and more significantly (P<0.0001) decreased it in the LD_{30}-treated females. However, the pattern of ecdysteroid production was the same in all three groups of insects (Fig. 3.1) (Perveen, accepted).

In the LD_{10}-treated females, during pre-vitellogenesis, the amount were (on the –4th day: 3.0±0.32 pg mg^{-1}; on the –3rd day: 3.5±0.8 pg mg^{-1}, after adult emergence), increased during vitellogenesis (on the –2nd day: 14.0±1.29 pg mg^{-1}; on the –1st day: 22.0±1.15 pg mg^{-1}; on the 0 day: 28.0±3.2 pg mg^{-1}, after adult emergence), peaked at the time of choriogenesis (on the 1st day after adult emergence: 46.0±3.2 pg mg^{-1}) and decreased when the insects started to deposit eggs (on the 2nd day after adult emergence: 9.0±1.66 pg mg^{-1}) and more significantly (P<0.0001) decreased thereafter (Fig. 3.1) (Perveen, accepted).

In the LD_{30}-treated females, during pre-vitellogenesis the amount was (on the –4th day: 2.5±0.92 pg mg^{-1}; on the –3rd day: 3.0±0.32 pg mg^{-1}, after adult emergence), increased during vitellogenesis (on the –2nd day: 9.5±0.93 pg mg^{-1}; on the –1st day: 17.0±3.39 pg mg^{-1}; on the 0

day: 23.0±0.8 pg mg^{-1}, after adult emergence), peaked on the time of choriogenesis (on the 1st day after adult emergence: 41.0±1.4 pg mg^{-1}) and decreased when the insects started to deposit eggs (on the 2nd day after adult emergence: 6.0±2.5 pg mg^{-1}) and more significantly ($P<0.0001$) decreased thereafter (Fig. 3.1) (Perveen, accepted).

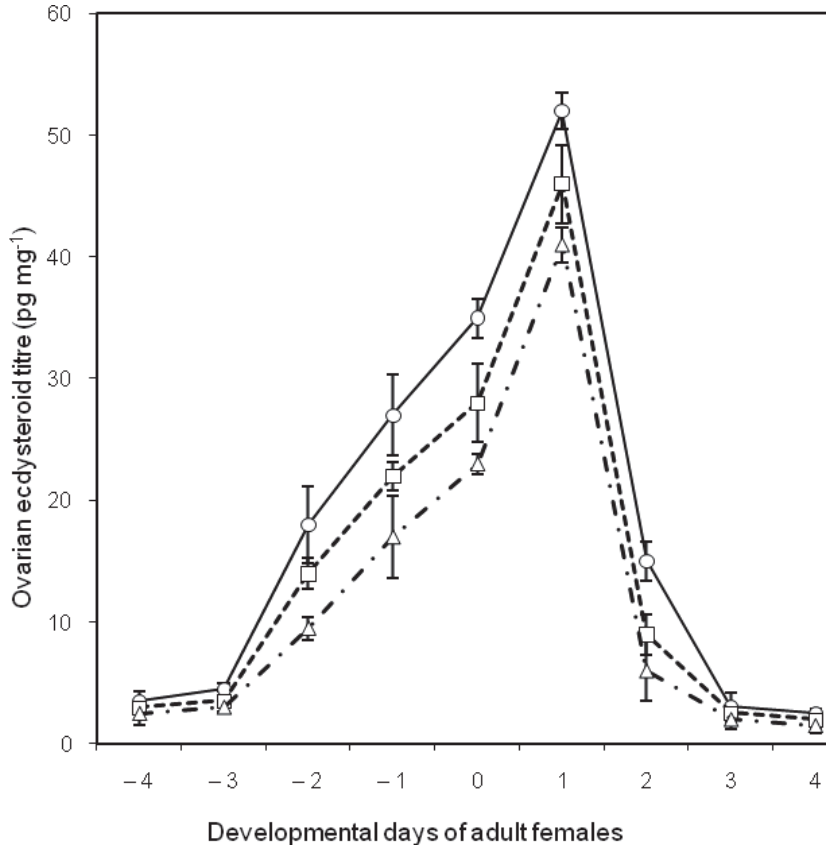


Fig. 3.1. Effect of sublethal doses of chlorfluazuron on the daily ovarian ecdysteroid titre of *Spodoptera litura* from 7 day-old female pupae to 4 day-old adult females; controls: O; LD_{10}: □; LD_{30}: Δ; data were analyzed by one-way ANOVA (Concepts, 1989) at $P<0.0001$ and followed by Scheffe's *F*-test (Scheffe, 1953) at 5%; vertical bars: SD; n=5 for each point; (Source: Perveen, accepted).

3.2 Effects on testicular constituents

3.2.1 Effects on testicular protein

Quantitative determination of the constituents of the testis of newly-emerged adult males showed that sublethal doses of chlorfluazuron significantly ($P<0.001$) reduced the amount of protein by the LD_{10}-treated and more significantly ($P<0.0001$) reduced by the LD_{30}-treated males compared with the controls measured in µg mg $testis^{-1}$ or µg $testis^{-1}$ (Table 3.7) (Perveen, 2000b).

In the control newly-emerged adult males, the amount of protein estimated was 0.95±0.03 µg mg testis^{-1} or 3.25±0.44 µg testis^{-1}. In the LD_{10}-treated newly-emerged adult males, it was 0.69±0.04 µg mg testis^{-1} or 1.87±0.29 µg testis^{-1}. In the LD_{30}-treated newly-emerged adult males, it was 0.46±0.08 µg mg^{-1} or 0.92±0.21 µg testis^{-1} (Table 3.7) (Perveen, 2000b).

Treatments[1]	n	Testicular Protein contents[2]	
		(M±SD) µg mg^{-1}	(M±SD) µg testis $^{-1}$
Control	15	0.95±0.03^{a}	3.25±0.44^{a}
LD_{10}	13	0.69±0.04^{b}	1.87±0.29^{b}
LD_{30}	11	0.46±0.08^{c}	0.92±0.21^{c}

[1]LD_{10}, 1.00 ng larva^{-1}; LD_{30}, 3.75 ng larva^{-1}; n: number of females used.
[2]Data were analyzed by one-way ANOVA (Concepts, 1989) and Scheffe`s F-test (Scheffe, 1953) at 5%. Means within a column followed by different letters are significantly different (P<0.0001).

Table 3.7. Effects of sublethal doses of chlorfluazuron on the testicular protein contents of newly-emerged adults after topical application to newly-ecdysed fifth-instars of *Spodoptera litura* (Source: Perveen, 2000b).

3.2.2 Effects on testicular carbohydrate

Quantitative determination of the constituents of the testis of newly-emerged adult males showed that sublethal doses of chlorfluazuron significantly (P<0.0001) reduced the carbohyderate content of the testis when considered in µg testis^{-1}, but the reduction was not significant (P<0.0001) when considered as µg mg testis^{-1} compared with the control (Table 3.8) (Perveen, 2000b).

In the control newly-emerged male adults, the amount of carbohydrate estimated was 1.70±0.25 µg mg testis^{-1} or 5.43 ±1.39 µg testis^{-1}. In LD_{10}-treated newly-emerged male adults, it was reduced by 10 % when considered per mg testis or by 26% when considered per testis. In LD_{30}-treated newly-emerged male adults, it was reduced by 14.7% when considered per mg testis or by 41.8% when considered per testis (Table 3.8) (Perveen, 2000b).

Treatments[1]	n	Testicular carbohydrate content[2]	
		(M±SD) µg mg^{-1}	(M±SD) µg testis^{-1}
Control	15	1.70±0.25^{a}	5.43±1.39^{a}
LD_{10}	13	1.53±0.19^{a}	4.02±0.53^{b}
LD_{30}	11	1.45±0.29^{a}	3.16±0.67^{b}

[1]LD_{10}, 1.00 ng larva^{-1}; LD_{30}, 3.75 ng larva^{-1}; n: number of females used.
[2]Data were analyzed by one-way ANOVA (Concepts, 1989) and Scheffe`s F-test (Scheffe, 1953) at 5%. Means within a column followed by different letters are significantly different (P<0.0001).

Table 3.8. Effects of sublethal doses of chlorfluazuron on the testicular charbohydrate contents of newly-emerged adults after topical application to newly-ecdysed fifth-instars of *Spodoptera litura* (Source: Perveen, 2000b).

3.2.3 Effects on testicular lipid

Quantitative determination of the constituents of the testis of newly-emerged adult males showed that sublethal doses of chlorfluazuron significantly (P<0.0001) reduced the lipid

content of the testis when considered in µg testis^{-1}, but reduction was not significant ($P<0.0001$) when considered as µg mg testis^{-1} compared with the control (Table 3.9). In the control newly-emerged adult males, the amount of lipid estimated was 11.01±0.63 µg mg testis^{-1} and 37.19±6.62 µg testis^{-1}. In the LD_{10}-treated newly-emerged adult males, it was reduced by 2.6%, when considered per mg testis and 25% when considered per testis. In the LD_{30}-treated newly-emerged adult males, it was reduced by 5.9%, when considered per mg testis, and 72.2% when considered per testis (Table 3.9) (Perveen, 2000b).

Treatments[1]	n	Testicular Lipid contents[2]	
		(M±SD) µg mg^{-1}	(M±SD) µg testis^{-1}
Control	15	11.01±0.63^{a}	37.19±6.62^{a}
LD_{10}	13	10.72±1.08^{a}	27.77±2.84^{b}
LD_{30}	11	10.36±0.62^{a}	20.97±2.15^{b}

[1]Source: Perveen, 2000b; LD_{10}, 1.00 ng larva^{-1}; LD_{30}, 3.75 ng larva^{-1}; n: number of females used.
[2]Data were analyzed by one-way ANOVA (Concepts, 1989) and Scheffe`s F-test (Scheffe, 1953) at 5%. Means within a column followed by different letters are significantly different ($P<0.0001$).

Table 3.9. Effects of sublethal doses of chlorfluazuron on the testicular lipid contents of newly-emerged adults after topical application to newly-ecdysed fifth-instars of *Spodoptera litura* (Source: Perveen, 2000b).

3.2.4 Effects on testicular DNA

Quantitative determination of the DNA of the testes of newly-ecdysed last-(sixth)-instar larvae through the 9th day after pupation to the 1st day after adult emergence showed that the concentration of DNA slowly and gradually increased until pre-pupation and remained constant until the 9th day after pupation (Fig. 3.2).

Sublethal doses of chlorfluazuron significantly reduced ($P<0.001$) the amount of DNA in LD_{10}-treated and more significantly ($P<0.0001$) reduced in LD_{30}-treated males compared with the control in the developmental stages described above measured in µg mg testis^{-1} (Fig. 3.2) (Perveen, accepted).

In the control newly-ecdysed last-(sixth)-instar larvae, the amount of DNA was 1.4±0.16 µg mg testis^{-1}. In the LD_{10}-treated larvae, it was 1.23±0.16 µg mg testis^{-1}. In the LD_{30}-treated larvae, it was 1.02±0.24 µg mg testis^{-1} (Fig. 3.2) (Perveen, accepted).

In the control for the 2nd day sixth-instar larvae, the amount of DNA was 3.52±0.26 µg mg testis^{-1}. In the LD_{10}-treated larvae, it was 3.24±0.21 µg mg testis^{-1}. In the LD_{30}-treated larvae, it was 2.88±0.38 µg mg testis^{-1} (Fig. 3.2) (Perveen, accepted).

In the control for the 4th day-sixth instar larvae, the amount of DNA was 6.42±0.31 µg mg testis^{-1}. In the LD_{10}-treated similar larvae, it was 5.04±0.21 µg mg^{-1}. In the LD_{30}-treated similar larvae, it was 3.90±0.27 µg mg testis^{-1} (Fig. 3.2) (Perveen, accepted).

In the control pre-pupae, the amount of DNA was 7.50±0.35 µg mg testis^{-1}. In the LD_{10}-treated similar larvae, it was 6.02±0.4 µg mg testis^{-1}. In the LD_{30}-treated similar larvae, it was 4.54±0.31 µg mg testis^{-1} (Fig. 3.2) (Perveen, accepted).

In the control on the 2nd day after pupation, the amount of DNA was 7.30±0.16 µg mg testis^{-1}. In the LD_{10}-treated similar pupae, it was 5.90±0.4 µg mg testis^{-1}. In the LD_{30}-treated similar pupae, it was 4.30±0.56 µg mg testis^{-1} (Fig. 3.2) (Perveen, accepted).

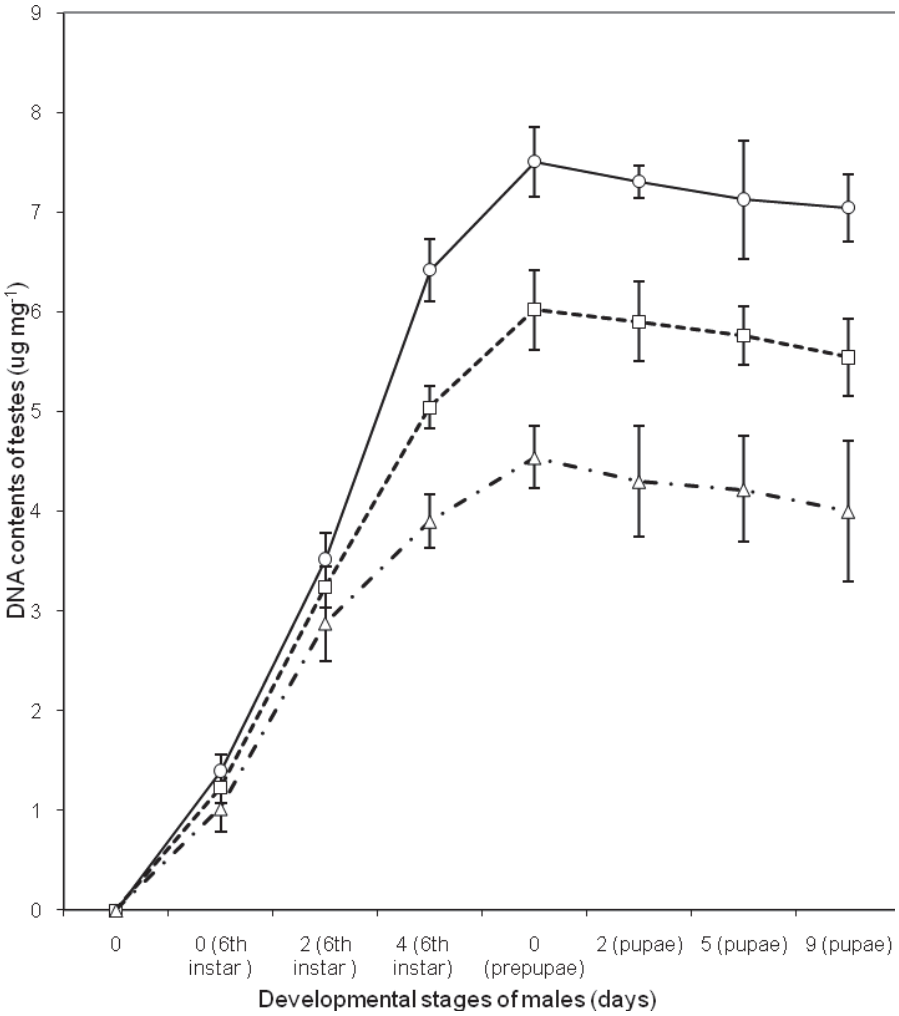


Fig. 3.2. Effect of sublethal doses of chlorfluazuron on the DNA contents of testes from newly-ecdysed sixth-instars to 9 day-old pupae of *Spodoptera litura*; controls: O; LD_{10}: □; LD_{30}: Δ; data were analyzed by one-way ANOVA (Concepts, 1989) at $P<0.0001$ and followed by Scheffe's *F*-test (Scheffe, 1953) at 5%; vertical bars: SD; L: larval, P: pupal and A: adult developmental days; n=5 for each point; paired larval testes and fused single pupal testis were considered as testes pair equivalent; (Source: Perveen, accepted).

In the control on the 5th day after pupation, the amount of DNA was 7.12±0.59 µg mg testis^{-1}. In the LD_{10}-treated similar pupae, it was 5.76±0.29 µg mg testis^{-1}. In the LD_{30}-treated similar pupae, it was 4.22±0.53 µg mg testis^{-1} (Fig. 3.2) (Perveen, accepted).
In the control on the 9th day after pupation, the amount of DNA was 7.04±0.34 µg mg testis^{-1}. In the LD_{10}-treated similar pupae, it was 5.54±0.39 µg mg testis^{-1}. In the LD_{30}-treated similar pupae, it was 43.2±0.71 µg mg testis^{-1} (Fig. 3.2) (Perveen, accepted).

When the DNA content was measured on the day before adult emergence, it slightly increased (7.52±0.9 µg mg^{-1}). Then, in newly-emerged adults, it sharply decreased (4.54 ± 0.44 µg mg^{-1}) and on the 1st day after adult emergence, it slowly decreased (3.52±0.49 µg mg^{-1}). In newly-emerged adults, the DNA content was 27.3% lowered compared with the LD_{10}-treated ones and 21% compared with the LD_{30}-treated ones. On the 1st day after adult emergence, it was decreased by 27.3% as compared to LD_{10}-treated ones and by 16. 6% compared with the LD_{30}-treated ones (Fig. 3.3) (Perveen, accepted).

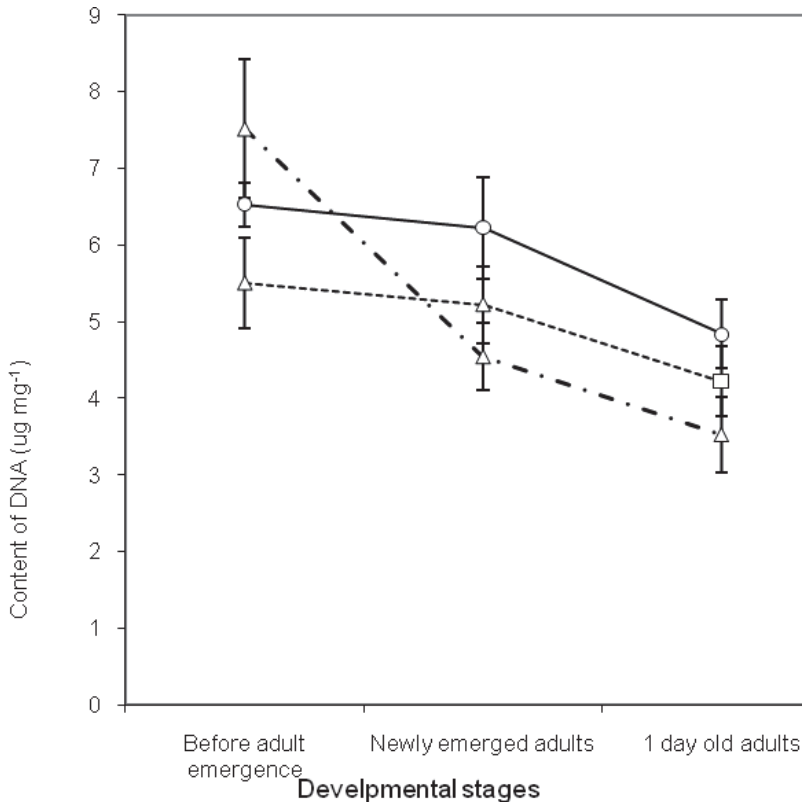


Fig. 3.3. Effects of sublethal doses of chlorfluazuron on the DNA contents of the testis over three developmental stages of *Spodoptera litura*; controls: O; LD_{10}: □; LD_{30}: Δ; data were analyzed by one-way ANOVA (Concepts, 1989) at $P<0.0001$ and followed by Scheffe's *F*-test (Scheffe, 1953) at 5%.; vertical bars: SD; n=5 for each point; (Source: Perveen, accepted).

However, in trend insects the tendency was quite different. In LD_{10}-treated specimen on the day before adult emergence, the DNA content was significantly decreased (by 13.3%) compared with the controls. In the same stage in the LD_{30}-treated specimen, it was significantly lowered (by 15.6%) those LD_{10}-treated ones. The same trend was observed in newly-emerged adults and on the 1st day after adult emergence of treated insects with both doses, i.e., in newly-emerged adults, it was decreased (6.2±2 6.6 µg mg^{-1}) from the day before adult emergence, but it was the greatest than the controls and greater than LD_{30}-

treated ones in which it was (5.22±0.5 µg mg^{-1}), which is greater than the controls. On the 1st day after adult emergence, it was decreased (4.84±0.45 µg mg^{-1}) than in newly-emerged adults and LD_{10}-treated 1 day-old adults, but it was greater than the controls (Fig. 3.3) (Perveen, accepted).

3.2.5 Effects on seminal vesicular DNA

When the DNA content was measured in the seminal vesicles on the day before adult emergence, it was 3.50±0.79 µg mg^{-1}. In newly-emerged adults, it increased to (4.52±0.42 µg mg^{-1}) and on the 1st day after adult emergence, it slightly increased to (4.72±0.30 µg mg^{-1}). However, in treated insects, the tendency was quite different. In the LD_{10}-treated specimen on the day before adult emergence, the DNA content was significantly decreased (by 20%) compared with the controls. For LD_{30}-treated ones, it was significantly decreased (by 27.9%) than the LD_{10}-treated ones. The same trend was observed in newly-emerged adults and 1 day-old adults treated with both sublethal doses compared with the controls. However, in newly-emerged LD_{10}-treated adults, it was almost constant on the day before adult emergence. On the 1st day after adult emergence LD_{10}-treated males, it had increased level compared with the newly-emerged adults of the similar treatment. Similar tendency was observed in LD_{30}-treated ones during the same adult developmental days as in LD_{10}-treatment (Fig. 3.4) (Perveen, accepted).

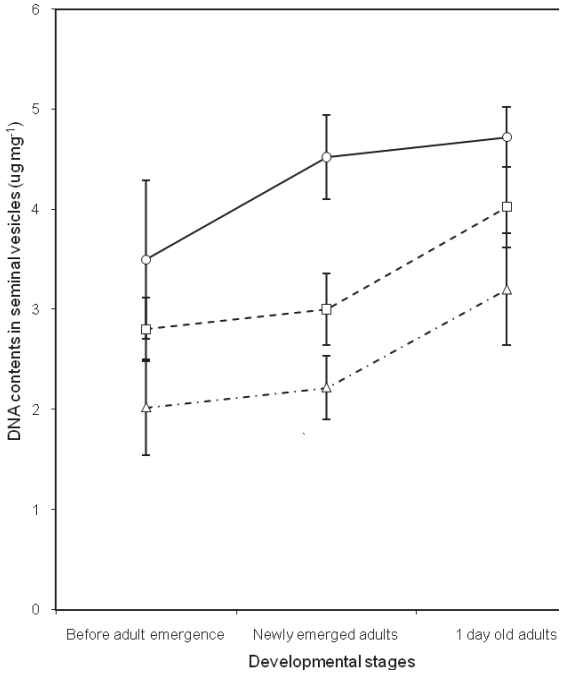


Fig. 3.4 Effects of sublethal doses of chlorfluazuron on the DNA contents of seminal vesicle over three developmental stages of *Spodoptera litura*; for controls: O; LD_{10}: □; LD_{30}: Δ; data were analyzed by one-way ANOVA (Concepts, 1989) at $P<0.0001$ and followed by Scheffe's *F*-test (Scheffe, 1953) at 5%; vertical bars: SD; n=5 for each point; (Source: Perveen, accepted).

3.2.6 Effects on aedeagular DNA

When the DNA content was measured in aedeagus to the 1 cm tube (area of collum formation of the cuticular secondary segment of the ductus ejaculatorious simplex) on the day before adult emergence, it was 1.20±0.16 µg mg^{-1} and then in newly emerged adults, it increased to 2.00±0.35 µg mg^{-1} and on the 1st day after adult emergence, it slowly increased to 2.50±0.37 µg mg^{-1}. However, in treated insects the tendency was a little changed. In the LD_{10}-treated insects on the day before adult emergence, the DNA content was significantly ($P<0.0001$) decreased (by 16.6%) compared with the controls. In the same stage in the LD_{30}-treated ones, it significantly decreased ($P<0.0001$; by 20%) compared with the LD_{10}-treated ones. The same trend was observed in newly emerged adults and on the 1st day after adult emergence of treated insects with both sublethal doses when compared with the controls. However, in both LD_{10}- and LD_{30}-treated newly emerged adults, the level slightly increased from before adult emergence but it sharply increased on the 1st day after adult emergence compared with the newly emerged adults (Fig. 3.5) (Perveen, accepted).

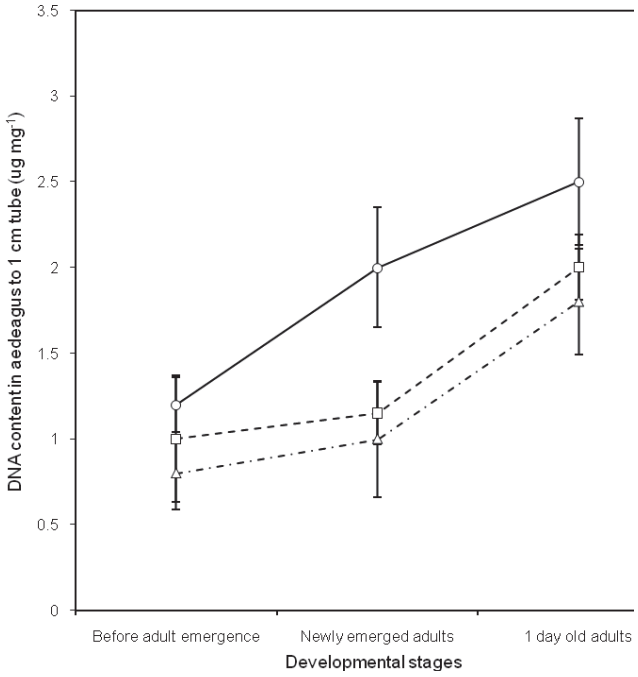


Fig. 3.5. Effects of sublethal doses of chlorfluazuron on the DNA contents of aedeagus to 1 cm tube over three developmental stages of *Spodoptera litura*; controls: O; LD_{10}: □; LD_{30}: Δ; data were analyzed by one-way ANOVA (Concepts, 1989) at $P<0.0001$ and followed by Scheffe's *F*-test (Scheffe, 1953) at 5%; vertical bars: SD; n=5 for each point; (Source: Perveen, accepted).

3.2.7 Effects on testicular RNA

Quantitative determination of the RNA content of the testis of newly-ecdysed sixth-instar larvae, pupae and male adults showed that the concentration of RNA was the greatest in larvae, decreased in pupae and again increased in adult males, the same as for DNA (Table 3.10) (Perveen, accepted).

Sublethal doses of chlorfluazuron significantly reduced ($P<0.001$) the amount of RNA in LD_{10}- and more significantly ($P<0.0001$) reduced in LD_{30}-treated males compared with the controls, measured in µg mg testis^{-1} or µg testis^{-1} in each developmental stage (newly-ecdysed sixth-instar larvae; newly-pupated pupae; newly-emerged male adults) (Table 3.10). In the control newly-ecdysed sixth-instars, the amount of RNA was 8.65±0.54 µg mg^{-1} and 16.32±0.82 µg testis^{-1}. In LD_{10}-treated larvae, it was reduced by 15.6% when considered per mg testis, and by 9.9%, when measured per testis. In LD_{30}-treated larvae, it was reduced by 37.6%, when considered per mg testis, and by 20.9%, when measured per testis (Table 3.10) (Perveen, accepted).

In the control newly-pupated male pupae, the amount of RNA was 8.01±0.49 µg mg testis^{-1} or 40.94±0.61 µg testis^{-1}. In the LD_{10}-treated newly-pupated male pupae, it was reduced by 14.5% when considered per mg testis, and by 4.5%, when measured per testis. In the LD_{30}-treated newly-pupated male pupae, it was reduced by 33.3%, when considered per mg of testis and by 5.4%, when measured per testis (Table 3.10) (Perveen, accepted).

In the controls newly emerged male adults, the amount of RNA was 9.33±0.76 µg mg^{-1} or 51.93±0.53 µg testis^{-1}. In the LD_{10}-treated newly-emerged male adults, it was reduced by 14.2%, when considered per mg testis and by 3.1%, when measured per testis. In the LD_{30}-treated newly-emerged male adults, it was reduced by 27.8% when considered per mg testis and by 7.7% when measured per testis (Table 3.10) (Perveen, accepted).

Treated stages	Treatments[1]	n	RNA contents[2] (M±SD) µg mg^{-1}	(M±SD) µg testis^{-1}
Larvae	Control	10	8.65±0.54^{a}	16.32±0.82^{a}
	LD_{10}	10	7.30±0.72^{b}	14.7±0.62^{b}
	LD_{30}	10	5.40±0.70^{c}	12.91±0.55^{c}
Pupae	Control	19	8.01±0.49^{a}	40.94±0.61^{a}
	LD_{10}	19	6.84±0.60^{b}	39.08±0.73^{b}
	LD_{30}	19	5.34±0.75^{c}	38.71±0.79^{c}
Adults	Control	19	9.33±0.76^{b}	51.93±0.53^{b}
	LD_{10}	19	8.01±0.51^{b}	50.30±0.91^{b}
	LD_{30}	19	6.74±0.50^{c}	47.94±1.14^{c}

[1]LD_{10}, 1.00 ng larva^{-1}; LD_{30}, 3.75 ng larva^{-1}; n: number of females used.
[2]Data were analyzed by one-way ANOVA (Concepts, 1989) and Scheffe`s F-test (Scheffe, 1953) at 5%. Means within a column followed by different letters are significantly different ($P<0.0001$).

Table 3.10. Effect of sublethal doses of chlorfluazuron on the RNA contents of the testes in different developmental stages after topical application to newly-ecdysed fifth-instars of *Spodoptera litura* (Source: Perveen, accepted).

3.2.8 Effects on testicular ecdysteroid titre

Measurment of the effects of sublethal doses of chlorfluazuron on the ecdysteroid titre of testis were conducted every 8 hours, from the 2 day-old sixth-instar larvae (0 hour) through to prepupae (88 hours) and after each 24 hours, from pupae (112 hours) to 2 day-old adults (376 hours). Sublethal doses were applied by same method as described for newly-ecdysed fifth-instar larvae of *S. litur* (as in Materials and methods). Ecdysteroid titre was measured in pg paired larval testes^{-1}; the fused single pupal testis was considered as testes pair equivalent (Perveen, accepted).

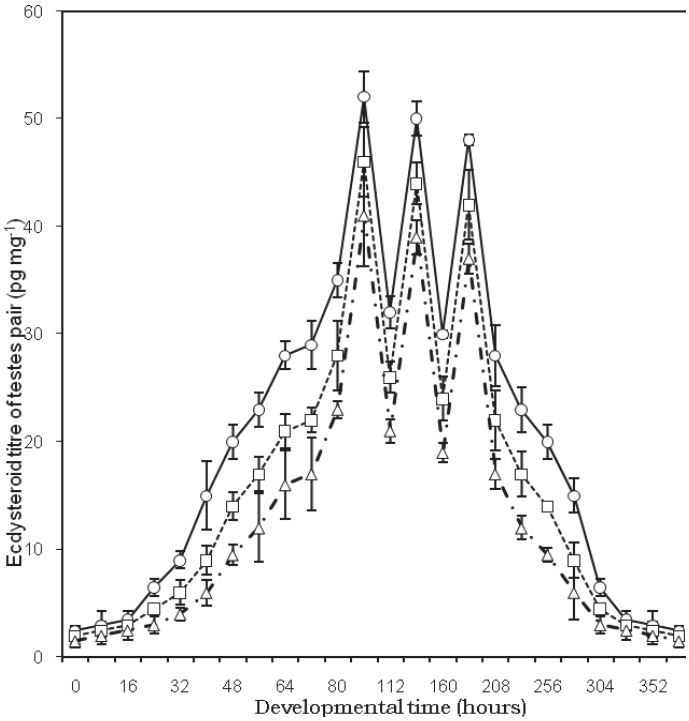


Fig. 3.6. Effects of sublethal doses of chlorfluazuron on the testicular ecdysteroid titre each consecutive 8 hours of *Spodoptera litura* from 2 day-old sixth-instars (0 hour) to pre-pupae (88 hours) and after each consecutive 24 hours from pupae (112 hours) to 2 day-old adults (376 hours); controls: O; LD_{10}: □; LD_{30}: Δ; data analyzed by one-way ANOVA at $P<0.0001$; after 16 hours all data are significantly different by Scheffe`s F-test at 5%.; vertical bars: SD; n=5 for each point; paired larval testes and fused single pupal testis were considered as testes pair equivalent (Source: Perveen, accepted).

Very low ecdysteroid titre was present in control newly-ecdysed sixth-instar larvae, when the testes were very small. It did not significantly increase until 16 hours of 2 day-old sixth-instar larvae. However, after 16 hours of 2 day-old sixth-instar larvae till the prepupal stage (80 hours), it significantly ($P<0.0001$) increased, as the testis increased in size and, simultaneously, spermatogenesis also increased and also when larval paired testes started to fuse to form a single testis (Fig. 3.6). Between 80 hours to 208 hours (5 day-old pupae), three

peaks of ecdysteroid titre were observed. The first peak was during 80–88 hours; the second one was at 88–136 hours; the third was at 136–208 hours old. At this time (third) spermatogenesis was at its peak and, simultaneously, sperm bundles were being transferred to the seminal vesicles. After that, the titre gradually decreased till 376 hours (2 day-old adults), (Fig. 3.6) (Perveen, accepted).

Sublethal doses of chlorfluazuron affected the ecdysteroid titre of testes during this all development time of the sixth-instar (last) larvae, pupae and adults. It was significantly ($P<0.001$) decreased by the LD_{10} and more significantly ($P<0.0001$) decreased by the LD_{30} during development of all stages of *S. litura*. However, in the LD_{10}- and LD_{30}-treated males the pattern of ecdysteroid titre production was the same as in the controls (Fig. 3.6) (Perveen, accepted).

4. Discussion

Effects of sublethal doses (LD_{10}: 1.00 ng larva^{-1}; LD_{30} : 3.75 ng larva^{-1}) of chlorfluazuron on biochemical were analysed to observe the causes of effects of chlorfluazuron on oÖgenesis and ovarian development (Perveen and Miyata, 2000; Perveen, 2009a); spermatogenesis and testicular development (Perveen, 2000b, 2009a); insemination (Perveen, 2008, 2009a), oviposition stimulating factors (Perveen, 2009b); and embryogenesis (Perveen, 2009c); and also ultimate effects on the fecundity, fertility and hatchability.

In the present research, a significant decrease in ovarian protein content in chlorfluazuron-treated females was observed (Table 3.1). Diflubenzuron also caused a decrease in ovarian protein content in *Cy. pomonella* (Soltani and Mazouni, 1992). Decrease in the ovarian protein content suggests interference by BPUs with vitellogenesis. In the current results, the decrease in ovarian protein content in chlorfluazuron-treated females was presumed to have several causes, such as the lack of protein in the ovarioles or interference of chlorfluazuron with the mechanism controlling yolk deposition. It has been reported that diflubenzuron could affect the protein content from other organs, such as the epidermis, in *T. molitor* (Soltani, 1984), ovaries in *C. pomonella* (Soltani et al., 1989a and b), and the concentration of haemolymph constituents in *T. molitor* (Soltani, 1990). In the control, newly emerged adult females, the amount of protein was 0.71±0.18 µg mg ovaries^{-1} and 53.9±18.5 µg pair ovaries^{-1}. However, in the LD_{10}-treated newly-emerged adult females, it was reduced up to 36.6% when calculated per mg ovaries and 46.6% per pair ovaries. In the LD_{30}-treated newly emerged adult females, it was reduced 40.8% when calculated per mg ovaries and 54.6% per pair ovaries (Table 3.1).

Maturation of insect testes also depends, among other factors, upon the materials that are taken up from the surrounding haemolymph and by materials synthesized by the testes *in situ*. These materials include protein, lipid and carbohydrate, all of which are required for development of the genital tract (Kunkel and Nordin, 1985; Kanost et al., 1990). In the present work, the decrease in testis protein content in chlorfluazuron-treated males may have several causes, such as lack of protein in the haemolymph of the males, and/or interference with the mechanism controlling spermatogenesis. In the control, newly emerged adult males, the amount of protein was 0.95±0.03 µg mg testis^{-1} and 3.25±0.44 µg testis^{-1}. In the LD_{10}-treated newly-emerged adult males, it was reduced up to 27.4% when calculated per mg of testis and 42.5% per testis. In the LD_{30}-treated newly-emerged adult males, it was reduced up to 51.6% when calculated per mg testis and 71.7% per testis (Table 3.7).

Barnby and Klocke (1987) found increased digestibility and reduced weight in azadirechtine-treated larvae of tobacco bud worm as a result of longer stay of protein in the gut and large accumulation of proteolytic enzymes. The reduction in larval weight and protein content was also observed in *H. armigera* larvae when fed on azadirechtine-treated diet (Javaid, 1989). The protein and peptide content were reduced in house fly larvae when fed on NFB- and nimocinol- (neem compounds) treated diets (Nizam, 1993). The present findings agree with those previous results. In the present work, sublethal doses of chlorfluazuron inhibited protein content in both ovaries and testes in newly emerged adults. IGR and other pesticides have been reported as protein inhibitors in various insect species, e.g. Chang et al. (1974), Philips and Loughton (1979), Ahmad and Naqvi (1985), Naqvi et al. (1986), Rizvi et al. (1986), Akhtar (1989), Javaid (1989), Naqvi et al. (1989), Masood (1990) Yasmeen et al. (1991) and Azmi (1993). Pesticides inhibit protein due to their poisonous effects. For examples, DDT, malathion, pyrethroids and IGRs reduced the protein content in susceptible strains of house flies, mosquitoes, stored grain pest and moths. In this work, reduction in protein content was observed in *S. litura* when sublethal doses were applied against newly-ecdysed fifth-instars. These results agree with the findings of previous workers. If differences are found, that may be due to differences in insect species or in methods or in IGR used.

Free amino acid composition of protein in the haemolymph of rice stem borer was affected by IGRs in the fat body (Chang et al., 1974). Ahmad and Naqvi (1985) reported on the toxic effect of dimilin against mosquitoes and flow rate of protein metabolites was affected. That may be due to changes at the molecular level. Grosscurt and Anderson (1980), Rizvi et al. (1986) and Hasan et al. (1987) reported changes in insect protein pattern after IGR treatment. Naqvi et al. (1986) reported similar results when cockroaches were treated with dimilin and penfluron. Naqvi et al. (1989) reported inhibition in the protein content of *Bl. germanica* and *M. domestica* larvae when treated with dimilin. Nizam (1993) reported 26% inhibition in protein content in *M. domestica* larvae when treated with dimilin. Similarly, in the present study, the protein content was reduced by the LD_{10} or LD_{30} doses in ovaries and testes. The percentage inhibition was greater than to Nizam (1993) results. This may be due to the different IGR used, application method or insect variations.

Activation instead of inhibition of some protein content was found in resistant strains of some insects (Naqvi et al., 1986; Akhtar, 1989). Nizam (1993) reported activation in malathion-treated larvae that were five-fold resistant to malathion, whereas IGRs and neem compounds inhibited protein content. However, in the present study, there was no activation but rather reduction in protein content in ovaries and testes. There is no report available that indicates activation of protein content in insects as result of application of chlorfluazuron. Diflubenzuron applied by dipping (10 mg ml^{-1}) at pupal ecdysis disturbed the development and also the changed the haemolymph titre of different carbohydrate metabolites. The pattern of haemolymphatic protein was also slightly affected after treatment (Soltani, 1990). In this study, the testis constituent of newly emerged male adults showed that sublethal doses of chlorfluazuron significantly reduced the amount of protein in the LD_{10}-treated and more significantly reduced in the LD_{30}-treated males compared with the controls, measured either in µg mg $testis^{-1}$ or µg $testis^{-1}$. However, the carbohydrate content of testes was not significantly lowered by these doses when measured in µg mg $testis^{-1}$ but compared with the controls were significantly lowered when measured in µg $testis^{-1}$. The lipid content of testes was not significantly lowered by these doses when measured in µg mg $testis^{-1}$. However, compared with the controls, they were significantly

lowered when measured in µg testis^{-1}. Moreover, these doses were reduced only protein contents but did not have effects on the charbohydrate and lipids contents in ovaries.

The proteins, lipids and carbohydrates present in the zygote of insects are a nutritional store for subsequent embryogenesis (Bownes et al., 1988). The morphological and temporal embryonic stages of *S. litura* affected by sublethal doses of chlorfluazuron applied topically to newly-ecdysed fifth-instars were allowed to description of effects of sublethal doses of chlorfluazuron exerted through eight embryonic stages during 0-84 hours of development of eggs after oviposition. In the current results, in chlorfluazuron-treated eggs, the decrease in protein content was presumed to have several causes, such as the lack of protein in the ovarioles development or interference of chlorfluazuron with the mechanism controlling yolk utilization. The protein-filled vesicles formed most of the volume of yolk in the oöcyte during oögenesis. Lipids were stored in insect eggs in the protein yolk bodies as well as in the triacylglycerol droplets (Telfer et al., 2009). Autoradiography of follicles labelled *in situ* with ^{3}H-glucose indicated that synthesis and deposition of glycogen were most rapid in the cortical cytoplasm of the oöcyte, with the assembled particles later moving into interstices between the lipid and protein yolk bodies deeper in the oöcyte (Mundall and Law, 1979). Presently, in chlorfluazuron-treated eggs, decreased in the protein, carbohydrate and lipid contents might be expected result of interference of chlorfluazuron in the similar mechanisms. The termination of vitellogenesis, a key step in late follicular development, normally occurred in the Cecropia moth, *Hyalophora cecropia* Linnaeus follicles when they had reached a length of 2 mm. But incubation in membrane-permeable analogues of cyclic adenosine monophosphate (cAMP) could induce the response in any vitellogenic follicle, regardless of its size (Wang and Telfer, 1996). The response was due to closure of the intercellular spaces (Wang and Telfer, 1997). Synthesis of the sulfated glycosaminoglycans deposited in the intercellular channels of the follicular epithelium was inhibited, water uptake causes the follicle cells to swell and close the emptied channels and tight junctions form between neighboring follicle cells. These were exactly the changes in nucleic acids exhibited by follicle cells during *in situ* termination of vitellogenesis (Wang and Telfer, 2000).

In the present work, the effect of chlorfluazuron on the DNA and RNA contents was estimated according to the method described by Burton (1956) and Munro (1966). The general growth of the insects is controlled by moulting hormone (MH) and juvenile hormone (JH) whose metabolism and degradation are checked by enzymes. If degradation continues then further action by that particular hormone will stop and ultimately growth of the insect is retarded. Later, it was suggested by Socha and Sehnal (1973), MH activated the synthesis of RNA and JH simultaneously induced the duplication of DNA. It means that growth hormones affect nucleic acid production and their quantity. A number of researchers (e.g. Attri and Ravi, 1980 a and b; Naqvi et al., 1989; Naqvi et al., 1993) worked on the aspect that IGR could act as a toxicant. They revealed a moderate to high level of inhibition of both nucleic acids. Philips and Loughton (1979) reported inhibition in RNA and protein synthesis. Sixty percent RNA inhibition was found after dimilin treatment. In the present work, both DNA and RNA were inhibited by the chlorfluazuron during ovaries, testes and egg development. However, inhibition was greater for DNA than RNA. Total RNA decreases rapidly whereas DNA content decreases steadily. Therefore, DNA was more sensitive to chlorfluazuron because it has longer half-life than dimilin. Chinzei and Tojo (1972) and Premkumar et al. (1991) described variations in DNA and RNA contents while studying them in *B. mori* and water scorpion, respectively. These reports are supporting by

the present findings. The percentage DNA and RNA was different after sublethal doses even when they were determined on same day of the same stage of *S. litura*. Sublethal doses of chlorfluazuron showed variation in their effects on DNA and RNA contents of ovaries and testes.

During mating sperm, were transferred from males by spermatophore into the bursa copulatrix of females. Sperm are stored here for a few hours and then, they are transferred into spermatheca of females, the depository organ for sperm storing, which consists of two parts, utriculus and legna. Mostly sperm are stored in utriculus, no sperm were observed in legna. The DNA content was estimated in utriculus after the 1st and 2nd matings. The DNA content in controls was 2.04±0.06 µg mg^{-1} and 1.75±0.08 µg mg^{-1} tissue after the 1st and 2nd matings, respectively, i.e., it was greater after first mating. However, after the second mating a greater number of sperm was transferred by the spermatophore. Perhaps more of the sperm were destroyed during travel from the *bursa copulatrix* to the *utriculus* of the spermatheca. However, significant reduction (1.25±0.09 µg mg^{-1} and 1.19±0.07 µg mg^{-1} after the 1st and 2nd mating, respectively) by the LD_{10} treatment and more significant reduction (0.85±0.08 µg mg^{-1} and 0.75±0.08 µg mg^{-1} after the 1st and 2nd mating, respectively) by the LD_{30} treatment were observed in DNA content compared with the controls. The reason was that DNA content is directly proportional to the number of sperm present in the testis. The number of sperm transferred to female during 1st and 2nd mating, in LD_{10}-treated *S. litura* significantly reduced and in LD_{30}-treated insects more significantly reduced (Perveen, 2008). Therefore, DNA content was also affected by sublethal doses (Table 3.5).

The DNA content in males significantly increased from newly-ecdysed sixth-instars until pre-pupae. Then, it remained constant from pre-pupae to before adult emergence (Fig. 3.2). After that in the control newly-emerged adults, it sharply and significantly decreased (Fig. 3.3). In I day-old adults, it decreased significantly but not very sharply. However, in LD_{10}-treated moth, the DNA content was significantly lowered and was even more significantly lowered in LD_{30}-treated moth compared with the controls (Figs. 3.2 and 3.3). However, the pattern was same from newly-ecdysed sixth-instars to before adult-emergence in both treatments. After that time variations were observed in both treatments compared with the controls, i.e. on the day of adult-emergence, the DNA content was significantly higher in treated insects compared with the controls. However, it was significantly lowered compared with the same treatment before adult-emergence. Again in 1 day-old adult, it was higher in treated insects compared with the controls; however, it significantly lowered compared with the same treatment (Figs. 3.2 and 3.3). These variations could be explained by the fact that the control newly-ecdysed sixth-instars to pre-pupae spermatogenesis was occurring and sperm were growing and developing, which required a greater quantity of DNA. Therefore, the DNA content significantly increased during these days. Then, from pre-pupae to before adult-emergence, the maturation of sperm took place, therefore, the DNA content did not significantly change but remained constant. After that, in control newly-emerged adults, sperm were transferred from the testis through the vas deferens to the seminal vesicles; therefore, the DNA content sharply and significantly decreased. This transfer of sperm continued till 1day-old adults. At that stage, it decreased significantly but not very sharply.

In LD_{10}-treated batches, the DNA content was significantly lowered and even more lowered still in LD_{30}-treated batches compared with the control during all larval development because of effect of chlorfluazuron. The pattern of decrease of DNA content was same from newly-ecdysed six-instars to before adult emergence in both treatments (Fig. 3.2) but different from controls. This was the time for spermatogenesis, growth and development of

sperm. After that, conditions were quite different in both treatments, i.e., on the day of adult emergence; the treatments significantly reduced the transfer of sperm from the testis through the vas deferens to the seminal vesicles. Therefore, the DNA content was significantly higher in the testis of treated insects compared with the control. However, it was significantly lowered compared with the same treatment before adult emergence (Fig. 3.3). Again, on the 1 day-old adults, the process of sperm transfer continued, therefore, the DNA content was increased in treated insects compared with the control. However, it was significantly lowered compared with the same treatment (Figs. 3.2 and 3.3).

In the seminal vesicles from before adult emergence to the 1 day-old adults of controls, the DNA content was continuously and significantly increased because sperm were transferred from the testis to the seminal vesicles through the vas deferens. Therefore, the DNA content sharply and significantly decreased in the testis. However, in the batches treated with both sublethal doses, it increased but the difference was not significant with treatments during adult development. In contrast, in the same batches of the testis, it significantly decreased. However, significant differences were observed with the LD_{10} and more significant differences with the LD_{30} compared with the controls over adult development. The reason was same, i.e. sperm were being transferred from the testis to the seminal vesicles but this was affected by the sublethal doses (Fig. 3.4).

When the DNA content of the control from *aedeagus* to the 1 cm tube was observed before adult emergence on the 1 day-old adults, it sharply and significantly increased because sperm were transferred from the seminal vesicles to this area. However, in batches treated with both sublethal doses, it was increased but the increase was not significant on the day of adult emergence because of treatments. But, it was significantly higher on the 1 day-old adults. Significant reduction with the LD_{10} treatment and even greater reduction with LD_{30} treatment were observed in the DNA content compared with the controls during adult development. The reason was that sperm were being transferred from the seminal vesicles to the aedeagus and to the 1 cm tube were affected by sublethal doses (Fig. 3.5).

The important point in the present results data was that both doses have a wide range of variations (Figs. 3.3-3.5) in their effect on DNA and RNA content. There were not only significant differences in DNA and RNA contents in the ovaries and testes when the treatments by both sublethal doses were compared with controls but significant differences were also observed between the sublethal doses (LD_{10} or LD_{30}) during different days of development of insect. It could be concluded that, primarily, chlorfluazuron has properties like other pesticides and, secondarily, it is a toxicant. That is why it is registered as one of the IGRs.

There are some previous reports on IGRs, effect on DNA and RNA. Philips and Loughton (1979) reported that actinomycine, dimilin (an IGR) and cyclohexamide inhibited RNA and protein synthesis in fourth instar nymphs of *L. migratoria*. About 60% inhibition of RNA was obtained by these compounds as compared with inhibition of RNA in the present work of about 3–8% by LD_{10} and 5–16% by LD_{30} doses in ovaries (Table 3.4) and 14–16% by LD_{10} and 27–38% by LD_{30} doses of chlorfluazuron in testes during different days of developmental stages which were observed (Figs. 3.2 and 3.3).

However, in the present work 19–50% inhibition of DNA was obtained as by LD_{10} and 41–83% by LD_{30} dose in ovaries (Fig. 3. 4) and 12–22% by LD_{10} and 27–43 %by LD_{30} dose in testis during different days of developmental stages which were observed (Figs. 3. 2 and 3.3). Saleem and Shakoori (1987) reported that the DNA content of *T. castaneum* remained unaltered when treated with sublethal doses (1.0 and 2.0 ppm) of permethrin. However, the

RNA content decreased 16% and 28% at these concentrations. Shakoori et al. (1988) reported that at higher doses of fenpropathrin, DNA and RNA contents were decreased up to 20% to 21%, respectively, in sixth-instar larvae of *T. castaneum*. Shakoori and Saleem (1989) reported that malathion was ineffective on the DNA and RNA content, but permethrin and malathion increased 28% and 27% the DNA and RNA activity after 120 hours of treatment. DNA increased up to 23% and 27% and RNA increased up to 14% and 18% by the pyrethroids, respectively. Tabassum (1994) agreed with earlier findings, where DNA and RNA were slightly inhibited by dimilin in *Tribolium* spp. In that case, dimilin inhibited by 33%, 21% and 32% the RNA content and by 44%, 50% and 43% the DNA content after 24, 72 and 144 hours, respectively, using a glass film method. Naqvi et al. (1992) reported nucleic acid inhibition in *M. domestica* when treated with NC and solfac.

Shakoori et al. (1985) reported that a sublethal dose (200 ppm) as well as a lethal dose (400 ppm) of a pyrethroid, fenpropathrin, against sixth-instar larvae of *T. castaenium* did not change the DNA and RNA content much under laboratory conditions. In another report of Saleem and Shakoori (1985) observed that the IGR, diflubenzuron, did not cause significant changes in DNA and RNA content in *Tribolium* spp. However, in the present research, the effects of sublethal doses (LD_{10} and LD_{30}) of chlorfluazuron on the inhibition of DNA and RNA content were observed and the inhibition of DNA and RNA were greater under for LD_{30} than LD_{10}. Thus, comparatively higher inhibition of DNA and RNA may be correlated with the fact that chlorfluazuron is a growth retardant.

In the present work, the highest inhibition level was observed after LD_{30} treatment by chlorfluazuron compared with the controls and the LD_{10} treatment which supports biodegradation of DNA concept (Stokes and Redfern, 1982; Jacobson et al., 1984). Different levels of inhibition may be due to different doses on female and male treatments (Table 3.4, 3.6 and 3.10; Figs. 3.2 and 3.3). DNA and RNA play an important part in living organisms and inhibition of nucleic acid consequently results in inhibition of proteins synthesis.

The DNA contents in testes was reduced significantly with LD_{10}-treatment and more significantly reduced with LD_{30}-treatment compared with the controls and the pattern of DNA contents was also the same, during larval development, however, the pattern of DNA was different during development of pupae and adults. The DNA contents were reduced in control as compared with LD_{10}- and LD_{30}-treatments. The RNA content in testes was reduced significantly with LD_{10}-treatment and more significantly reduced with LD_{30}-treatment compared with the controls, throughout larvae, pupae and adults. Nucleic acids synthesized in the zygote play an inductive role in blastulation and gastulation during early embryogenesis (Czihak and Horstadius, 1970). Recent results of significant decrease in nucleic acids in chlorfluazuron-treated eggs might be interfere in the same mechanism may clarify in future. Like many other insects, lepidopteron provision their eggs with high concentrations of ecdysteroids that were made available to developing embryos and prehatching larvae (Hoffman et al., 1980). The inhibition of DNA and RNA by chlorfluazuron in the present studies was higher than reported by Saleem and Shakoori (1985) and Shakoori et al. (1988). The higher inhibition may be because chlorfluazuron is an IGR. The growth disruption and abnormal growth caused by chlorfluazuron may be due to inhibition of nucleic acids and consequently, inhibition of protein synthesis. However, to confirm this needed to do more research work in this connection.

4.1 Effects on ecdysteroid titres of ovaries and testes

Ecdysone not only controls the ecdysis but also plays an important role in development and maturation of the reproductive system. It is not only produced by the prothoracic glands but

is also produced by the ovaries and testes themselves as well as their sheaths (Loeb et al., 1984; Loeb et al., 1986a and b; Gelman et al., 1988). It has been reported that diflubenzuron could affect ecdysteroid secretion from other organs such as: the epidermis in *T. molitor* (Soltani, 1984), ovaries in *C. pomonella* (Soltani et al., 1989a and b), and the concentration of haemolymph constituents in *T. molitor* (Soltani, 1990). In the mosquito, *A. aegypti*, the ovary begins to produce ecdysone after a blood meal. Ecdysone, after being converted to 20-hydroxyecdysone, stimulates the synthesis and secretion of vitellogenin by the fat-body. The vitellogenin is taken up by the growing oöcytes and becomes part of the yolk (Hagedorn et al., 1979). In the present research, in *S. litura*, according to current evidences would tend to suggest that ecdysteroid does not play the same role as described by Hagedorn and co-workers. Ecdysteroid titre was observed in 7 day-old pupae to 4 day-old adult female ovaries.

Soltani and Mazouni (1997) reported ecdysone levels that when ovaries from 4 day-old females of mealworm, i.e. at the end of vitellogenesis were cultured in media supplemented with diflubezuron, preliminary data indicated that ovarian amounts of ecdysteroids, as measured *in vivo*, changed during vitellogenesis of the *T. molitor* in a characteristic way. The amounts were low during pre-vitellogenesis, increased during vitellogenesis, peaked at the time of choriogenesis, and decreased when the insects started to deposit eggs and thereafter. Diflubezuron at the two tested doses (5 and 10 µg ml^{-1} medium) significantly decreased the amounts of ecdysteroid produced *in vitro* by ovaries. In contrast, the neurohormone stimulated the *in vivo* production of ecdysteroids by the ovaries. The neurohormone stimulated the *in vivo* production of ecdysteroid by the ovaries. In the present research, the ecdysteroid titre in the ovaries was determined after each consecutive 24 hours on the 7 day-old female pupae to 4 day-old adult females. Preliminary data from the controls indicated that the ovarian ecdysteroid, as measured *in vivo*, changed during vitellogenesis of *S. litura*, in the same characteristic way as reported in the meal worm. Chlorfluazuron at the two tested sublethal doses significantly affected the amounts of ecdysteroid produced *in vivo* by the ovaries. It significantly lowered in the LD_{10}-treated females and even more significantly lowered in the LD_{30}-treated females. However, the pattern of the levels of ecdysteroid titre secretion was the same as in the controls (Fig 3.1).

Spermiogenesis of the spermatocysts in the diapausing pupal testis of *M. brassica* was investigated *in vitro* with reference to ecdysteroids released from the testis. The quantity of the ecdysteroids and activity of spermiogenesis induction were measured in a medium conditioned with the culture of testes for 1 to 6 days (Shimizu et al., 1985). In the present research, ecdysteroid titre was observed in testes of *S. litura*. In the early development of last-(sixth)-instar larvae (from 0 to 16 hours), when the testes were very small, the ecdysteroid titre of the testes was very low and remained unchanged. As the testes increased in size and, simultaneously, spermatogenesis increased, the titre significantly increased until 80 hours. After 80 hours, when the larval paired testes fused to form a single testis, the titre was significantly reduced until the pre-pupal stage. Eighty hours after ecdysed of sixth-instars to 208 hours (5 day-old pupae), three peaks of ecdysteroid titre were observed. It is suggested that this times was the peak times of spermatogenesis. At 208 hours, the different developmental stages of sperm were present (Fig. 3.6).

The function of testis ecdysteroid is still open to speculation. However, ovarian and testicular ecdysteroids were important for the development and maturation of the reproductive tracts and play an important role in oögenesis and spermatogenesis. The

ecdysteroid, 20-hydroxyecdysone, is believed to be necessary for maximal spermatocyte mitosis and resumption of sperm development after diapause as well as testes fusion and genital tract maturation in lepidopterans (Nowock, 1972, 1973). Ecdysteroid titre in the present research suggest it, might perform the same function as described above. The testes of *H. virescens* and *L. dispar* were examined *in vitro* for the ability to produce ecdysteroids. Production was detected in the testes removed from larvae at mid- and late-periods in last-instar larvae, in the testes from pupae after the 3rd day of pulpal development and in testis taken from young male adults (Loeb et al., 1984). In the present research, the testes of *S. litura* were examined *in vivo* for the ability to produce ecdysteroid. Production was detected in the testes removed from sixth-instar larvae from 0 hour to 88 hours (pre-pupae) after each consecutive 8 hours and in testis from pupae (112 hours) to 2 day-old adults (376 hours) after each consecutive 24 hours. It was observed that very low ecdysteroid titres were present in the control newly-ecdysed sixth-instars. The titre was not significantly increase until 16 hours of 2 day-old sixth-instars. However, after 16 hours of 2 day-old sixth-instars till pre-pupae (80 hours), it was significantly increased. After 80 hours to 208 hours (5 day-old pupae), it was also significantly increased with three peaks of ecdysteroid titre observed in the testis. After that it slowly decreased till 376 hours (2 day-old adults) (Fig. 3.6).

It was the testis sheaths rather than the content that was physiologically active for the production of ecdysteroid (Gelman et al., 1988). Gonadal ecdysteroid can stimulate the production of growth factor from the sheath which in turn, promotes the growth, development and maturation of the genital tract (Loeb et al., 1996). Tissues from the irregular testis sheath and its extensions that form the follicle wall were responsible for the production of ecdysteroids (Loeb, 1986). In the present study there was might be the same source of secretion of ecdysteroid and they performed the same functions as described by Gelman et al. (1988) and Loeb et al. (1986, 1996). Soltani et al. (1989a and b) reported that dipping newly-ecdysed *T. molitor* pupae in an acetone solution of diflubenzuron prevented most of them (73%) from completing development. Such blocked insects did not secret the adult cuticle and remained apolysed. Their ecdysteroid level analysed by radioimmunoassy was not increase. However, injection of 20-hydroxyecdysone (2–10 µg) several days after diflubenzuron application allowed the secretion of a new cuticle but with an abnormal architecture but with a high content in N-acetyl-amino sugar as revealed by fluorescent wheat germ agglutinin (Soltani et al., 1984a and b). The effects of diflubenzuron were observed on the larval-larval and larval-pupal ecdysis cycles of *T. molitor*, after treatment at ecdysis. In both cases, the first part of the cycle, from ecdysis to apolysis was apparently not affected, but the pharate periods were lengthened; treated insects were generally unable to perform ecdysis and died. The ecdysteroid titres in the haemolymph of treated insects were measured with a radio-immunoassay and compared with the controls. During larval-larval cycles, the single ecdysteroid increase was not affected by the diflubenzuron treatment. In the present research, the effect of sublethal doses of chlorfluazuron on ecdysteroid in the ovaries and testes were determined. The titres were significantly lowered in LD_{10} and even more significantly lowered in the LD_{30} treated insects compared with the controls, from the 7 day-old female pupae to the 4 day-old female adults after each consecutive 24 hours in the ovaries and during the 2 day-old sixth-instar larvae (0 hour) to pre-pupae (88 hours) after each consecutive 8 hours and from pupae (112 hours) to 2 day-old adults (376 hours) after each consecutive 24 hours in the testes during life-span of *S. litura* (Figs. 3.1 and 3.6).

During larval-pupal development, a significant difference was observed; whereas two ecdysteroid peaks occurred in the controls; but the first peak was not modified (Soltani et

al., 1989a and b). During the present work, between 80 hours to 208 hours (5 day-old pupae), three peaks of ecdysteroid titre were observed in testes. The first peak was between 80 – 88 hours; the second between 88–136 hours and between third or last was between 136–208 hours old. After that, the titre slowly decreased (Figure 3.6). However, the ecdysteroid titre in testes was reduced significantly with LD_{10}-treatment and more significantly reduced with LD_{30}-treatment compared with the controls. Therefore, these reductions were responsible for reduction in different parameters of spermatogenesis and testicular development reported by Perveen (2000b).

Ecdysteroids control vitellogenesis and egg maturation in pharate adult females of the Indian meal moth, *Plodia interpunctella* (Hubner) (Shirk et al., 1990). They include ecdysone and 20-hydroxyecdysone, as well as hydrophilic conjugates of these and other ecdysteroids in both the silk moth, *Bombyx mori* Linnaeus (Ohnishi and Chami, 1977) and H. *cecropia* (Rubenstein et al., 1986). Assays of individual follicles in *Hyalophora* ovarioles showed that the ecdysteroids accumulate during both vitellogenesis and post-vitellogenic water uptake. In greater wax moth, *Galleria mellonella* Linnaeus the total accumulation was equivalent to 74 µg of ecdysone gram of eggs^{-1} (Hsiao and Hsiao, 1979). That 20-hydroxyecdysone directly or indirectly triggers aspects in ovarian development became apparent during the metamorphic moults (Ohnishi et al., 1977). In the present research, the ecdysteroid titre was continuously increased in untreated eggs throughout the embryogenesis till hatching with the great variations during 0–84 hours of development (stages 1–8). When segmentation and blastulation were started during 0–4 hours (stages 1–2), a very low peak of ecdysteroid was observed. After that three peaks of ecdysteroid were observed which were correlated with events during embryogenesis. Initially, in the 32 hour old egg (at the late stage 6), when two head positions, the frontal and lateral positions were marked, the first lowest peak of ecdysteroid was appeared. Secondarily, in the 64 hour old egg (at the late stage 7), when organogenesis was established and the embryo was appeared larva-like, the middle peak of ecdysteroid was appeared. Finally, in the 84 hour old egg, when the embryo resembled the first instar; head darkness, appendages of the head segments, tracheal tubules and limbs were visible and the larva was ready to hatch at the late stage 8, the last highest peak of ecdysteroid was appeared.

It has been reported that DFB could affects ecdysteroid secretion from other organs, such as the epidermis, in the yellow mealworm, *Tenebrio molitor* Linnaeus (Soltani 1984), ovaries in *C. pomonella* (Soltani et al., 1989a and b), and the concentrations of haemolymph constituent in *T. molitor* (Soltani, 1990). In chlorfluazuron-treated eggs, the LD_{10} significantly ($P<0.05$) and LD_{30} more significantly ($P<0.05$) reduced the ecdysteroid titres during development of eggs (Fig. 3.10). However, the patterns of the ecdysteroid titres were the same as in untreated ones (Fig. 3.10). Moreover, the first and second peaks appeared 4 hours later in LD_{10}-treated and 8 hours later in LD_{30}-treated eggs compared with untreated ones (Fig. 3.10). Therefore, sublethal doses of chlorflazuron were extended their effects on embryogenesis of *S. litura* through reduction ecdysteroid titre in egg' contents during the eight embryonic developmental stages of progenies in F_1 generation.

5. Summary

In *Spodoptera litura*, sublethal doses of chlorfluazuron (LD_{10}: 1.00 ng larva^{-1} or LD_{30}: 3.75 ng larva^{-1}) topically applied at newly-ecdysed fifth-instars of *S. litura*, reduction in parameters of oogenesis (Perveen and Miyata, 2000), spermatogenesis (Perveen, 2000b), insemination

(Perveen, 2008) and embryogenesis (Perveen, 2009c) were ultimate effects of reduction of biochemical of the ovaries and testes which were analysed here with reference to effects of sublethal doses of chlorfluazuron. The same doses of chlorfluazuron significantly reduced the protein, DNA, RNA contents and ecdysteroid titre with no effects on the carbohydrate and lipid contents in ovaries during developmental days of females, which resulted in reduction of ovarian weight, length of ovarioles, thickness of follicular epithelium, number of mature ova, cell density germarium and size of basal oöcytes. The ovarian ecdysteroid titre, measured *in vivo*, changed during vitellogenesis in a characteristic way: the titre was low during pre-vitellogenesis, increased during vitellogenesis and peaked at choriogenesis. Sublethal doses also significantly reduced the protein, carbohydrate, lipid, DNA, RNA contents and ecdysteroid titre during developmental days of males, by effecting size and weight of testis, thickness of testis sheath, size and number of eupyrene and apyrene sperm bundles. During testis development, three peaks of ecdysteroid titre were observed. The second peak was appeared at 88–136 hours when larval paired testes started to fuse to form a single testis. Sublethal doses also effects insemination by reducing spermataphore weight, number of inseminated sperm. Sublethal doses also significantly reduced the protein, carbohydrate, lipid, DNA, RNA contents and ecdysteroid titre during embryogenesis. Three peaks of ecdysteroid titre were observed which were correlated with events during embryogenesis. The last highest peak was appeared at 84 hours when larva was ready to hatch at the late stage 8. The embryo resembled the first instar; head darkness, appendages of the head segments, tracheal tubules and limbs were visible. However, patterns of reduction in protein, carbohydrate, lipid, DNA, RNA contents and ecdysteroid titre were the same in all tests. Finally, collective effects of all parameters have been resulted reduction in the fecundity, fertility and hatchability of *S.litura.*

6. References

Ahmed, S. O. and Naqvi, S. N. H. (1985). Toxicity and effects of dimilin on the protein pattern of *Aedes aegypti* (PCSIR strain). Proc. Entomol. Soc. Karachi. 14–15: 121–132.

Akhtar, K. (1989). Determination of DDT and malathion resistance in *Musca domestica* (L.) of Karachi region and their effects on protein and enzyme patterns. *Ph. D. Thesis, Department of Zoology, University of Karachi.* p. 250.

Attrib, S. and Ravi, P. G. (1980a). Studies on the pesticidal value of neem oil by-products. Pestology. 4: 16–20.

Attrib, S. and Ravi, P. G. (1980b). Neem oil extract: an effective mosquito larvicide. Indian J. Entomol. 42: 371–374.

Azmi, M. A. (1993). Determination of DDT, malathion and solfac (cufluthrin10%) resistance against various strains of *Culex fatigans* and their effects on enzymatic and protein patterns. Ph. D. Thesis, Department of Zoology, University of Karachi. pp. 280.

Barbosa, P. (1974). Dissecting fluids and saline solutions. Manual of basic techniques in insect' histology (ed. by A.D. Mass), p. 243. Oxford Publisher, London, UK.

Barnby, M. A. and Klocke, J. A. (1987). Effects of azadirachtin on the nutrition and development of tobacco budworm, *Heliothis virescens*. J. Ins. Physiol. 33: 69–75.

Bownes, M., Shirras, A., Blair, M., Collins, J., and Coulson, A. (1988). Evidence that insect embryogenesis is regulated by ecdysteroids released from yolk proteins. Proc. Natl. Acad. Sci. U.S.A. 85: 1554–1557.

Bradford, M. M. (1976). A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein-dye-binding. Annal. Biochem. 72: 248 – 254.

Burton, K. (1956). Method for the determination of nucleic acids. J. Biochem. 62: 300–315.

Chang, C. C. and Borkovec, A. B. (1980). Effects of diflubezuron and penfluron on viability of house fly eggs. Ann. Entomol. Soc. Amer. J. Econ. Entomol. 73: 285–287.

Chang, S. C., Borkovec, A. B. and Braun, B. H. (1974).

Chemosterilant activity of antineoplastic agents. Trans. NY. Acad. Sci. II (36): 11–107.

Chinzei, Y. and Tojo, S. (1972). Nucleic acid changes in the whole body and several organs of the silkworm, *Bombyx mori* during metamorphosis. J. Ins. Physiol. 18: 1683–1698.

Concepts, A. (ed.) (1989). Super ANOVA. p. 120. Berkeley Publisher, Pamldale, California, USA.

Czihak, G., and Horstadius, S. (1970). Transplantation of RNA-labeled micromeres into animal halves of sea urchin embryos. A contribution to the problem of embryonic induction. Devel. Biol. 22: 15–30.

Duchateau, G. and Florkin, M. (1959). Sur la tres halosemie des insectes et sa signification. Arch. Intl. Physiol. Biochem. 67: 306–314.

Gelman, D. B., Woods, C. W. and Borvokovec, A. B. (1988). Ecdysteroid profile for haemolymph and testes from larvae, pupae, and pharate adults of the European cornborer, *Ostrinia nubilalis*. Arch. Ins. Biochem. Physiol. 7: 267–279.

Gilbert, L. I., Bollenbacher, W. E., Goodman, W., Smith, S. L., Agui, N., Granger, N. A. and Sedlak, B. J. (1980). Hormones controlling insect metamorphosis. *Recog.Prog. Hor. Res. 36*: 401–449.

Goldschmidt, R. (1916). The function of the apyrene spermatozoa. Sci. *44*: 544–546.

Goldsworthy, G. J., Mordue, W. and Guthkelch, J. (1972). Studies on insect adipokinetic hormones. Gen. Comp. Endocrinol. 18: 545–551.

Grosscurt, A. C. and Anderson, S. O. (1980). Effect of diflubenzuron on some chemical and mechanical properties of the elytra of *Leptinotarsa decemlineata.* Proc. Kon. Nederland Acad. Van Weten Schappes Series C. U. 83:143–149.

Hagedorn, H. H., Shapiro, J. P. and Hanaoka, K. (1979). Ovarian ecdysone secretion is controlled by brain hormone in an adult mosquito. Nature 282: 92 – 94.

Hasan, A., Ahmed, I., Nizam, S. and Naqvi, S. N. H. (1987). Insect growth regulator activity of diflubenzuron and its effects on the protein pattern of *Halys dentatus* (Fabr.) (Hemiptera: Pentatomidae). Pak. J. Entomol. 2: 51–57.

Hoffman J, Lageaux M, Hertru C, Charlet M, Goltzene (F.1980). Ecdysone in reproductively competent female adults and embryos in insects. *In:* Progress in Ecdysone Research, Biomedical Research. (edited by J. Hoffman) pp. 431-465. Elsevier, North Holland.

Horn, D. H. S., Wilkie, R. B., Sage, B. A., and O'Connor, J. D. (1976). A high affinity antiserum specific for the ecdysone nucleus. J. Ins. Physiol. 24: 901–903.

Hsiao T, Hsiao D. (1979). Ecdysteroids in the ovary and eggs of the greater wax moth. J. Ins. Physiol. 24: 45-52.

Jacobson, M., Stokes, J. B., Warthen, J. D. Jr., Redfern, R.E., Reed, D.K., Webb, R. E. and Telek, L. (1984). Neem research in the U. S. Department of Agriculture: an update. Proc. 2nd Intl. Neem Conf. (1983) 31–42.

Javaid, M. A. (1989). Biochemical investigation on the effect of azadirachtin on development of *Heliothis armigera* (Hub.). M.Phil. Thesis, Quid-e-Azam University, *Islamabad.* p. 120.

Kanost, M. R., Kawooya, J. K., Law, J. H., Ryan, R. O., Heusden, V. M. C. and Ziegler, R. (1990). Insect haemolymph proteins. *In: "Advances Insect physiology"* (edited by P. D. Evans and V.B. Wigglesworth) v. 22, p. 299–396. Academic Press, London.

Kunkel, J. G. and Nordin, J. H. (1985). Yolk proteins. *In:* "Comprehensive Insect Biochemitry, Physiology and Pharmacology" (edited by G. A. Kerkut and L. I. Gilbert) v. 1, p. 83–111. Pergamon Press, Oxford.

Le Bras, S. and Echaubard, M. (1977). Variations quantitatives des proteines dans les tissues de la mouche domestique (*Musca domestica* L.) au course de la vie imaginale, apres intoxication par 1'Hempa Bull. Soc. Zool. France. 102: 95–105. (Abstract in English read)

Loeb, M. J. (1986). Ecdysteroids in testis sheaths of *Heliothis Virescens* larvae: An immunological Study. Arch. Ins. Biochem. Physiol. 3: 173–180.

Loeb, M. J., Bell, R. A., Gelman, D., Kochanesky, J., Lusby, W. and Wagner, R. M. (1996). Action cascade of an insect gonadotropin, testes edysiotropin, in male Lepidoptera. Invert. Rep. Develop. 303: 181–190.

Loeb, M. J., Brand, E. P. and Birnbaum, M. J. (1984). Ecdysteroid production by testes of budworm, *Heliothis virescens*, from last-larval instar to adult. J. Ins. Physiol. 30: 375–381.

Loeb, M. J., Brand, E. P., Giebultowicz, J. M. and Wood, C.W. (1986a). Endocrine regulation of the testes and of spermatogenesis in insects. IV Intl. Symp. Invert. Rep. Lille, France. p. 182.

Loeb, M. J., Brand, E. P. and Wood, C. W. (1986b). Effects of exogenous ecdysteroid titre on endogenous ecdysteroid production *in vitro* by testes of the tobacco budworm, *Heliothis virescens*. J. Exp. Zool. 240: 75–82.

Masood, N. (1990). Esterase activity and associated insecticide resistance in the red flour beetle (*Tribolium castaneum*). M.Phil. Thesis, Quid-e-Azam University, Islamabad. pp. 250.

Minitab, Inc. (1979). Statistics software. Release 12. Minitab Inc., Stat College, USA. p. 16801.

Mitsuhashi, J. (1995). A continuous cell line from pupal ovaries of the common cutworm, *Spodoptera litura* (Lepidoptera: Noctuidae). Appl. Entomol. Zool. 30: 75–82.

Mokrasch, L. C. (1954). Analysis of hexose phosphates and sugar mixtures with the anthrone reagent. J. Biol. Chem. 208: 55–59.

Munro, H. N. (1966). The determination of nucleic acids. *In:* "Methods of biochemical analysis" (edited by D. Glick, ed.) v. 14, p. 113–176. Insecticide publisher, New York, USA.

Naqvi, S. N. H., Ali, S. M., Khan, M. F., Tabassum, R. and Azmi, M. A. (1992). Determination of comparative efficacy of neem fractions (RB-a) and Coopex, against grasshoppers in the field and laboratory. Proc.12th Cong. Zool. pp. 132.

Naqvi, S. N. H. and Rub, A. (1985). Effect of dimilin on the activity of melanization enzyme of *Musca domestica* L. (PCSIR strain). J. Sci. Karachi Univ. 13: 171–177.

Naqvi, S. N. H. and Perveen, F. (1993). Toxicity of some plant extracts in comparison to Coopex (Bioallethrin: Permethrin) against stored grain pest (*Callosobruchus analis*) (Coleoptera: Tenebrionidae). Pak. J. Entomol. 8: 5–15.

Naqvi, S. N. H. and Perveen, F. (1991). Toxicity and residual effect of *Nerium indicum* crude extract as compared with Coopex against adults of *Tribolium castaneum* (Coleoptera: enebrionidae). Pak. J. Entomol. 6: 35–44.

Naqvi, S. N. H., Shafi, S. and Zia, N. (1989). Histochemical localization of proteins and alkaline phosphatases in diflubenzuron treated insects. Pak. *Sci. Res. 32*:190–193.

Naqvi, S. N. H., Shafi, S. and Zia, N. (1986). Effects of diflubenzuron and penfluron on the protein pattern of *Blattella germanica.* Pak. J. Entomol. 1: 81–86.

Nizam, S. (1993). Effects of allochemicals against 3rd instar larvae of *Musca domestica* L. (Malir strain). *Ph. D.* Thesis, Department of Zoology, University of Karachi. pp. 250.

Nowock, J. (1973). Growth and metamorphosis in the testes of *Ephestia Kulniella*, *in vitro*. J. Ins. Physiol. 19: 941–949.

Nowock, J. (1972). Induction of Imaginal differentiation by ecdysone in testes of *Ephestia kulniella.* J. Ins. Physiol. 8: 1699–1704.

Ohnishi E, Mizuno T, Chataani F, Ikekawa M, Sakurai S. (1977). 2-deoxy-alpha-ecdysone from ovaries and egg of the silkworm *Bombyx mori*. Sci. 197: 66–67.

Ohnishi, E. and Chami, F. (1977). Biosynthesis of ecdysone in the isolated abdomen of the silkworm, *Bombyx* mor*i*. Develop. Grow. Diff. 19: 67–70.

Omatsu, M., Yoshida, K. and Toki, T. (1991). Development of malformed larvae induced by a benzoylphenyl urea insecticide chlorfluazuron in the common cutworm, *Spodoptera litura* Fabricius. Pestic. Sci. 16: 189– 194.

Perveen, F. (2011) Effects of sublethal doses of chlorfluazuron on the testicular biochemical constituents of *Spodoptera litura* (Lepidoptera: Noctuidae). Afr. J. Biotech. (Accepted).

Perveen, F. (2011) Effects of sublethal doses of chlorfluazuron on the ovarian biochemical constituents of *Spodoptera litura*. Journal of Chemical Society of Pakistan (accepted).

Perveen, F. (2009a). Effects of chlorfluazuron on the reproduction of *Spodoptera litura*. (ed. by AG Müller), p. 184. VDM-Verlag Publisher, Berlin, Germany.

Perveen, F. (2009b). Effects of residual chlorfluazuron on haemolymph-borne oviposition-stimulating factors in *Spodoptera litura*. Entomol. Experiment. Appl. 132: 241–249

Perveen, F. (2008). Effects of sublethal doses of chlorfluazuron on insemination number of inseminated sperm in the common cutworm, *Spodoptera litura* (F.) (Lepidoptera: Noctuidae). J. Entomol. Sci. 11: 111–121.

Perveen, F. (2006). Reduction in egg hatch after a sublethal dose of chlorfluazuron to larvae of the common cutworm, *Spodoptera litura*. Physiol. Entomol. 31: 39–45.

Perveen, F. (2000a). Sublethal effects of chlorfluazuron on reproductivity and viability of *Spodoptera litura* (F.) (Lep., Noctuidae). J. Appli. Entomol. 124: 223–231.

Perveen, F. (2000b). Effects of sublethal dose of chlorfluazuron on testicular development and spermatogenesis in the common cutworm, *Spodoptera litura*. Physiol. Entomol. 25: 315–323.

Perveen, F. and Miyata, T. (2000). Effects of sublethal dose of chlorfluazuron on ovarian development and oogenesis in the common cutworm, *Spodoptera litura* (F.) Lepidoptera: Noctuidae). Ann. Entomol. Soci. Amer. 93: 1131–1137.

Philips, D. R. and Loughton, B. G. (1979). The effect of inhibition of RNA and protein synthesis on the development of larvae of *Locusta migratoria*. Insect Biochem. 2: 241–245.

Premkumar, D. R. D., Jane, E. P. and Mathavan, S. (1991). Biochemical changes during embryonic development in the aquatic hemipteran bug, *Laccotrephes grisens*. Ins. Biochem. 21: 381–388.

Rees, H. H. and Isaac, R. E. (1985). Biosynthesis and metabolism of ecdysteroid and method of isolation and identification of the free and conjugated compounds. Meth. Enzymol. IIIB. 03: 377–437.

Retnakaran, A., Macdonald, A., Nicholson, D. and Cunningham, J. P. (1989). Ultrastructural and autoradiographic investigations of the interference of chlorfluazuron with cuticle differentiation in the spruce bud worm, *Choristoneura fumiferana*. Pestic. Biochem. Physiol. 35: 172–184.

Rizvi, S. A., Yasmeen, N., Zaidi, R. H. and Ahmad, S. O. (1986). Toxicity and effect of juvenile hormone analogue (methoprene) on the protein of *Oxyrhachus tranda* (Fabr.) (Homoptera: Membracidae). Pak. J. Entomol. 1: 7–16.

Rubenstein E, Kelly T, Culbert C, Woods C, Weeks B. 1986. Ecdysteroids in developing ovarian follicles of *Hyalophora cecropia*. Arch. Ins. Biochem. Physiol. 3: 253–263.

Saleem, M. A. and Shakoori, A. R. (1985). Comparative effects of permethrin and malathion on DNA, RNA and total protein contents in *Tribolium castaneum* adults. Pak. J. Zool. 18: 395–402.

Scheffe, H. (1953). A method for judging all contrasts in the analysis of variance. Biometrika *40*: 87–104.

Schmidt, G. and Thannhauser, S. I. (1945). A method for the determination of deoxyribonucleic acid, ribonucleic acid and phosphoprotein in animal tissues. J. Biol. Chem. 161: 83–92

Schneider, W. C. (1957). Determination of nucleic acid in tissues by pentose analysis. In: "Method in Enzymology" (edited by S.P. Colowick and N. O. Kaplan) v. 3, p. 680–684. Academic press, New York, USA.

Shakoori, A. R. and Saleem, M. A. (1989). Some macromolecular abnormalities developed by the interaction of malathion and permethrin and subsequent refeeding in *Tribolium castaneum* larvae. Arch. Ins. Biochem. Physiol. 11: 203–215.

Shakoori, A. R., Fayyaz, M. and Saleem, M. A. (1988). Biochemical changes induced by fenpropathrin in the 6th instar larvae of *Tribolium castaneum* (Herbst.) (Coleoptera: Tenebrionidae). J. Store. Prod. Res. 24: 215–220.

Shakoori, A. R., Fayyaz, M., Saleem, M. A. and Ali, S. S. (1985). Toxicological studies on a recently developed synthetic pyrethroid, fenpropathrin against *Tribolium castaneum* (Herbst.) larvae. *6th Pakistan Cong*. Zool. p. 36–37.

Shimizu, T., Moribayashi, A. and Agui, N. (1985). *In vitro* analysis of spermatogenesis and testicular ecdysteroids in the cabbage armyworm, *Mamestra brassicae* L. (Lepidoptera: Noctuidae). Appl. Entomol. Zool. 20: 56–61.

Shirk P, Bean D, Brookes V. (1990). Ecdysteroids control vitellogenesis and egg maturation in pharate adult females of the Indian meal moth, *Plodia interpunctella*. Arch. Ins. Biochem. Physiol. 15: 183–199.

Socha, R. and Sehnal, F. (1973). Inhibition of insect development by simultaneous action of prothoracic gland hormone and juvenile hormone. J. Ins. Physiol. 19: 1449–1453.

Soltani, N. (1984). Effects of ingested diflubenzuron on the longevity and peritrophic membrane of adult mealworms (*Tenebrio molitor* L.). Pestic. Sci. 15: 221–225.

Soltani, N., Delachambre, J. and Delbecque, J. P. (1989a). Stage-specific effects of diflubenzuron on the ecdysteroid titres during the developmental of *Tenebrio molitor*: Evidence for a change in hormonal source. Gen. Comp. Endocrinol. 76: 350–356.

Soltani, N., Mauchamp, B. and Delbeque, J. P. (1989b). Effects of diflubenzuron on the ecdysteroid titres in two insect species. Akad Landwirtsch Wiss. Tag.Ber., DDR, Berlin. 274: 171–177.

Soltani, N. and Mazouni, N. S. (1997). Diflubenzuron and oögenesis in the codling moth, *Cydia pomonella*. Pestic. Sci. 34: 257–261.

Soltani, N. and Mazouni, N. S. (1992). Oögenesis in mealworms: cell density of germarium, thickness of chorion and ecdysteroid production. Effects of regulators. Med. Fac. Landbouww. Univ. Gent. 62: 565–571.

Soltani, N. (1990). Action du diflubenzuron et de la 20-hydroxyecdusone sur les glucides et les proteins hemolymphtiques chez les nymphes de *Tenebrio molitor* L.(Coleoptera: Tenebrionidae). Ann. Soc. Ent. Fr. 26: 575–584.

Stokes, J. B. and Redfern, R. E. (1982). Effect of sunlight on Azadirachtin antifeeding potency. J. Environ. Sci. Health. 17: 57–65.

Tabassum, R. (1994). Toxicity and residual effects of some neem compounds (Nimocinol etc) in comparison with IGR (Dimilin) against store grain pests. *Ph.D. Thesis,* Department of Zoology, University of Karachi. pp. 350.

Telfer, W. H., Rubenstein, E. and Pan, M. L. (1981). How the ovary makes yolk in *Hylophora. In:* "Regulation of Insect Development and Behaviour" (edited by M. K. Wroclaw) p. 637–654. Technical University Press, London, UK.

Walpole, R. E. and Myers, R. H. (1998). Probability, statistics for engineer scientist, 6th eds. Prentice-Hall, USA.

Williams, C. M. (1952). Physiology of insect diapause. IV. The brain and prothoracic glands as an endocrine system in the *Cecropia* silk. Biol. Bull. 103: 120–138.

Wang Y, Telfer W. (1996). Cyclic nucleotide-induced termination of vitellogenin uptake by *Hyalophora cecropia* follicles. Ins. Biochem. Mole. Biol. 26: 85–94.

Wang Y, Telfer W. (1997). Cyclic nuclotide-stimulated termination of vitellogenesis in *Hyalophora cecropia*: formation of a diffusion barrier and the loss of patency. J. Ins. Physiol. 43: 675–684.

Wang Y, Telfer W. (2000). Cyclic nucleotide-dependent protein phosphorylation in vitellogenic follicles of *Hyalophora cecropia*. Ins. Biochem. Mole. Biol. 30: 29–34.

Yasmeen, N., Asdaque, S. T., Nurulain, S. M., Jafri, S. M. H., Jahan, M., Khan, M. F. and Naqvi, S. N. H. (1991). Determination of toxicity of methoprene (ZR-515) against *Halys dentatus* Fabr. and *H. qadri* Abbasi and Ahmad and its effect on protein pattern. Pak. J. Entomol. 6: 63–71.

14

Insecticide Treatment and Physiological Quality of Seeds

Lilian Gomes de Moraes Dan[1], Hugo de Almeida Dan[1], Alessandro de Lucca e Braccini[1], Alberto Leão de Lemos Barroso[2], Thiago Toshio Ricci[1], Gleberson Guillen Piccinin[1] and Carlos Alberto Scapim[1]

[1]*Department of Agronomy, State University of Maringá, Maringá, Paraná,*
[2]*Department of Agronomy, University of Rio Verde, RioVerde, Goiás, Brazil*

1. Introduction

The use of high-quality seeds enhances the probability of success of a crop. Another important factor is the use of techniques which target the improvement of seed field performance. Applications (seed dressing) of fungicides, inoculants, insecticides and micronutrients on seeds are practices most used by farmers for several years. These products have provided more favorable conditions to crop's growth as well as its development.

Chemical treatment on seeds have been one of the most common techniques in use on current farming due to its low-cost technology, low-environmental impact, and, in general, a significant effect on yield (Zambolim, 2005).

High quality seed is one of the essential prerequisites to achieve higher crop productivity. Seed physiological quality is influenced by genetic traits inherited from their parent trees, as well as germination and vigor, which are affected by environmental conditions, harvesting methods, drying process, processing, storage and packing (Zambolim, 2005).

Nevertheless, storage of insecticide treated seeds may cause significant reduction on both germination and vigor as demonstrated by a number of authors (Bittencourt et al., 2000); (Gotardo et al., 2001). This fact might be related to deterioration caused by the use of insecticides, which induce the formation of free radicals and subsequent oxidative stress (Braguini, 2005).

Chemical treatment on seeds has become an important procedure on agricultural production by a number of reasons. Firstly, by using this kind of treatment, many pest insects attacking not only seeds but also in some cases the aerial part of plants can be efficiently controlled. Secondly, these products can be handled in either protected or controlled environment regardless of climatic conditions, therefore, reducing additional and unwanted machinery movement in the land for cultivation.

These arguments are added to the fact that, in that referred kind of treatment; small quantities of products are used per area unit, which implies lower risks of environmental contamination. In addition, chemical treatment is a procedure of simple implementation and low cost (Goulart, 1999; Machado, 2000).

Silva (1998) stated that, when treating seeds, one can protect the plant during germination and young-seedling stages which are phases of greater susceptibility. This treatment aims to ensure seeds' full performance by achieving the desired planting density.
Nevertheless, evidences obtained from some studies have shown that some seed dressing products, under certain situations, may cause reduction in germination as well as seedling survival due to the phytotoxic effect. Significant vigor decreases were induced by carbofuran in corn seeds after treatment and a subsequent 30-day-period storage (Bittencourt *et al.*, 2000). Corn seeds treated with the insecticides, such as deltametrine and pirimiphos-methyl in high doses (Fessel *et al.*, 2003) lowered longevity, vigor and emergency speed of seedlings. Moreover, corn seeds treated with the insecticide phipronil caused reductions in seedlings radicular growth (Silveira *et al.*, 2001).
On the other hand, Barbosa *et al.* (2002), when treating bean seeds with the insecticides imidacloprid and thiamethoxam, verified that the active ingredients provided some improvement in the crop's agronomical traits, as a result, increasing productivity. Barros *et al.* (2005) verified greater percentage on bean seeds germination in treatments using the insecticide phipronil. However, Tavares *et al.* (2007) observed no difference on germination and vigor when using different doses of thiamethoxam in soybean seed treatment.
Some seed companies have been adopting the anticipated seed treatment process before bagging or when handing seeds over to the producer. Some issues have been discussed by Menten (1996) regarding the use of anticipated treatment. One of them is related to a possible phitotoxic effect which might be enhanced due to long period of seed storage after treatment. However, due to the lack of information within literature, it is not possible to assure whether insecticides can influence on seeds' germination and vigor, mainly during storage. Therefore, this study will focus on the discussion of previous studies regarding the effects of insecticides on seeds' quality in crops such as soybean and corn when treated and then stored.

2. Importance of physiological quality of seeds

Quality is an important aspect in seed production and, thus, must be considered as a standard of excellence. Nevertheless, quality, regarding its functional aspect and in a broad sense, must be considered as either a specification or a group of specifiers within certain limits or tolerances which must be met (Lima, 2003; Ferreira and Borghetti, 2004).
Seed quality is defined as the set of all genetic, physical, physiological and sanitary attributes which affect the capacity to output high-productivity plants. Physical quality comprises both physical purity and physical condition of seeds. Physical purity of seeds - known as seeds' purity - is characterized by the proportion of physical components in seed lots, such as pure seeds, weed seeds, other crop seeds as well as inert matter. Physical condition is characterized by moisture degree, size, color, density, appearance, mechanical damage and also damages caused by insects and infections caused by diseases (Popinigis, 1985).
The level of physiological quality of seeds is assessed by two fundamental parameters: viability and vigor. Viability is measured mainly through germination test in order to determine seed's maximum germination under favorable conditions.
Vigor detects more subtle changes in physiological quality not revealed by the germination test. Seeds' vigor is the reflection of a group of characteristics which determines its physiological potential, that is, the ability to present appropriate performance when exposed to different environmental conditions (Marcos Filho, 1999).

A successful crop implantation is fundamentally important to obtain yield enhancements. This success, most of the time, is achieved by using high quality seeds (Rocha Junior, 1999). Thus, during crop production process, the adoption of either advanced sowing techniques or crop tillage methods are not enough because the quality of seeds to be used is characterized as a primary factor in the further establishment and development of the crop.

Low quality seeds generally show diminished germination and vigor, originating heterogeneous crop and lessening crop population. According to Resende *et al.* (2003), high crop yield depends directly on the quality of seeds used for sowing.

Seeds' quality have been assigned to its physical purity, high-genetic potential, high germination and vigor, absence of mechanical damage, good health and uniformity of seed size (Lima, 1997).

According to Delouche (1975), seed quality involves several attributes including: cultivar's genetic purity, which is important to the development of the crop, as well as its uniformity, mainly to maturation; physical purity which consists of seeds free from inert matter, weed seeds and other crops; germination, in which high quality seeds should present germination above 85%; vigor, in which viable seeds from a lot must be sufficiently vigorous in order to rapidly and uniformly emerge under the most varied soil conditions and quickly develop into productive plants.

In accordance to Peske *et al.* (2003), the assessment of seeds physiological quality – sowing and lots commercialization purposes – has been ultimately based on the germination test, which is of practical usefulness for this end. Its methodology is standardized and its results reproduced intra- and inter-laboratories.

It's also important to take into account that seeds' initial quality is affected by a number of factors, such as vigor of parent plants, climatic conditions during maturation, maturation stage at harvesting, mechanical damage degree and drying (Carvalho & Nakagawa, 1988). Special attention should be taken to avoid seeds' potential reduction during storage. During storage, seeds present varying quality levels due to what has happened during development in the field (Yamada, 1989).

Moreover, during storage, they are subjected to deterioration in detriment of interactions between physical, chemical and biological processes represented mainly by temperature, humidity and availability of oxygen, microorganisms, insects, rodents and birds (Santos & Mantovani, 1997).

3. Seed treatment with insecticides and storage

Among the hindrances of farming is the loss of crop productivity caused by pest insects which – in agrosystems – find favorable conditions to its development due to monoculture practices over broad areas.

The number of pests attacking seeds and plants in early stages in several crops has also significantly increased, as a result, causing losses in the initial stand. Soil and aerial part pests have caused significant losses, leading to the need of a preventive treatment by using insecticides. Some of the insecticides available in the market present systemic action, which promote control in the initial phase of development, avoiding crop spraying in the early 20 days.

In this context, insecticides contribute significantly to the increase of yield, exerting control over these organisms (pests or pathogens). Although there are other methods or strategies of

control, insecticides application has been the most employed method due to its easy employment, fast achievement of results and lack of other equally efficient methods. These insecticides comprise an essential tool for current farming (Castro, 2005).

In order to avoid possible losses associated with the actions of insects, soil and aerial pests which may attack seeds and young plants, the preventive use of insecticides is an alternative practice in seeds' treatment (Silva, 1998). This practice, when appropriately accomplished, can reduce the number of foliar applications, which, many times must be started right after seedlings emergence (Menten, 1991).

Insecticides used in seeds' treatment differ from those applied in traditional pulverizing methods by their systemic action in the plant. In the soil, they detach from the seeds and, due to their low- pressure steam and aqueous solubility, they are slowly absorbed by the roots, granting the plant an appropriate period of protection against soil and aerial part pests (Silva, 1998).

Brazilian pesticides industry, propelled by the excellent agricultural performance, tripled its income in the last decade. The Brazilian market, which was US$ 947 million in 1992, reached US$ 3.1 billion in 2003, and in 2008 closed US$ 5.15 billion. In 2003, the Brazilian market was listed as the top three in the world rank, according to Cristiano Walter Simon, president of the National Association of Vegetal Defense. In addition, in 2008, the Brazilian pesticides industry became the largest in the world in sales revenue, overcoming the United States. Insecticides comprise 27.5% of this market, attesting its real importance (MAPA, 2009).

In the late 80s', concerns regarding the indiscriminate use of agrotoxics provoking damages to rural workers and the environment were intensified. Findings on new groups of insecticides, less toxic and aggressive to the environment, have corroborated to lessen the problem (EMBRAPA, 2002).

Thus, seed treatment is a preventive farming method, which consists of the application of insecticides on seed's surface, not only to control pests during storage, but equally important, to protect seedlings during germination and the initial period of crop establishment. In most countries where farming is intensive and highly productive, treatment is accomplished basically in their own seed processing units. The use of phytosanitary products applied via seeds is a common practice among many crops, nevertheless, growing concern regarding environment and safety during the process of those seeds' manipulation have increased the demands for new application technologies, which might reduce risks without jeopardizing seeds' quality.

Controlling pest insects in the phase prior to the implementation of a crop or at sowing time means that seeds' treatment is considered one of the most recommended measures in farming, providing less use of pesticides and avoiding environmental pollution problems (Machado, 2000).

The application of phytosanitary products aims to permit seed germination through accomplishing pest control and to protect seeds from soil pests. As a result, seed treatment by using phytosanitary products has become a frequent practice in order to assure an appropriate emergence and initial development of seedlings in the field (Novembre & Marcos Filho, 1991). Carrying out chemical treatment, most of the time, can avoid crop re-sowing, according to evidences of Goulart et al. (1995) and Menten (1996).

Therefore, 95% of the volume of soybean seeds in Brazil is commercialized with some kind of chemical treatment (Pereira, 2005). Besides insecticide application, other products can take part in the treatment, changing it into means for technology transferring. Incorporation of micronutrients, biostimulants and fungicides are among these practices (Fossati, 2004).

The use of insecticides on seeds is a technique which has been arisen due to an increase of insects' attack on the cycle onset because with direct sowing, these pests are sheltered either in straw or soil, causing damages to the newly deployed crop. Thus, insecticides industry for seed treatment has been fast-growing. Nowadays, around 30% of soybean seeds have been treated with this product (Baudet & Peske, 2006).

Within a number of problems faced in the production and conservation of seeds, the biggest one refers to pests during storage, in which losses of the stored product – due to this attack – represent a high percentage, whereas an adequate storage would prevent the damage of a great deal of production of many crops in Brazil (Carvalho, 1978).

Besides qualitative (contamination, nutritional value degradation, propagation and fungi development) and quantitative (mass reduction) depletions, pest attack to seeds can cause losses of both germinative power and vigor (Padilha & Faroni, 1993).

Some restrictions were discussed by Menten (1996) regarding the utilization of seeds' treatment with chemicals. Among them, cited by the author, the possible cytotoxic effect intensified after treatment, can lead to a reduction on germination and vigor of seeds (Ginasi *et al.*, 2000).

Although insecticide seed treatments have been considered one of the most efficient methods for using this kind of pesticide (Gassen, 1996; Ceccon *et al.*, 2004), results on studies have revealed that some products, when applied to seeds may also cause germination and seedling survival reduction. (Oliveira & Cruz, 1986; Kashypa *et al.*, 1994; Nascimento *et al.*, 1996).

In corn crops, significant depletion in seed vigor were observed by Bittencourt *et al.* (2000) after cabofuran application, similarly acknowledged by Fessel *et al.* (2003) who reported reduction in longevity, vigor and seedling emergence speed of corn crops after treating seeds with deltametrine and pirimiphos-methyl in high doses. Silveira *et al.* (2001) observed a smaller seedling radicular development after applying the insecticide phipronil on seeds.

When assessing seedling hight after treating black bean seeds, Guimarães *et al.* (2005) observed the negative effects of the insecticides carbofuran, thiamethoxam and imidacloprid. Nevertheless, the two latter ones only presented toxicity if seeds were previously treated from ten and thirty days and from five and ten days prior to sowing, respectively.

In soybean crops, Dan *et al.* (2010), when assessing some seed treatment insecticides over diferent storage periods, observed that results obtained from seed germination (Figure 1) indicated that, except for thiamethoxam, all of the other treatment insecticides significantly reduced seed germination throughout storage compared to the witness. Tavares *et al.* (2007) observed no difference on both germination and vigor when using different doses of thimethoxam in soybean seed treatment. Similarly, Barros *et al.* (2001) observed no reduction on germination compared to the witness when using thimethoxam on bean crop.

At time zero (Figure 1), it can be noticed that all insecticide treatments reached an appropriate germination level for soybean seeds, with germination percentages above 80% – minimum value referred by Brasil (2005) – characterizes the absence of harmful effects under this variable at the time of sowing on the date of seed treatment.

A reduction in seed germination percentage can be observed as and when storage period increases after insecticide treatment (Figure 1), particularly for acephate and carbofuran. Acephate was the insecticide which showed a greater reduction in seed germination during storage periods, providing a reduction of 0.55 percentage points (pp) in germination for each day of seed storage. These results indicate that this insecticide provoked a negative

effect on soybean seed germination and that this effect linearly increased according to the extension of storage period, in accordance to what was similarly reported by Oliveira and Cruz (1986) in corn seeds.

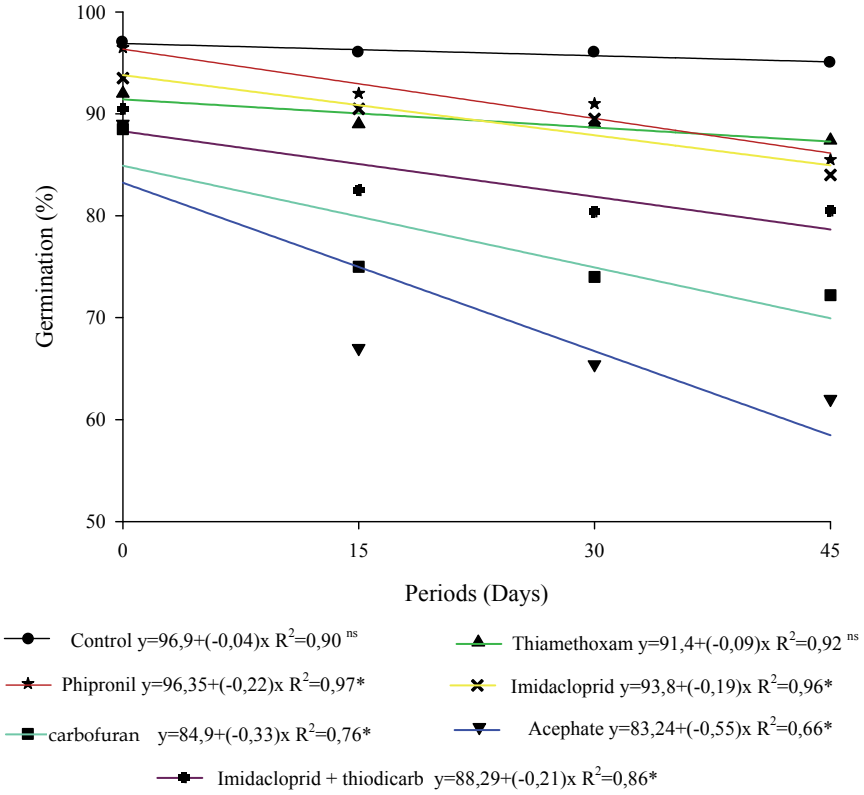


Fig. 1. Germination (%) of soybean seeds after insecticide treatments in four periods of storage (Dan *et al*., 2010).

Carbofuran-based insecticide seed treatment followed the same behavior of that one of the acephate, however, presenting a smaller reduction on seed germination when compared to the latter. It is important to remember that germination is an essential process in order to guarantee a good final plant stand. Thus, soybean seeds treated with acephate and carbofuran when stored may result – at the sowing time – in plant stand failures, and, consequently, in crop yield drops (Dan *et al*., 2010).

Nevertheless, it can be observed that at the end of storage period (45 days), treatments using the insecticides such as phipronil and thimethoxam. Imidacloprid and imidacloprid plus thiodicarb still presented germination percentage above 80% (Figure 1).

When assessing insecticide treatment in soybean seeds (Dan *et al*., 2011), during short storage periods observed that (Figure A), except for thiamethoxam, phipronil and imidacloprid, all the other insecticide treatments significantly reduced seed germination throughout storage when compared to the witness.

After up to three days of storage, all insecticides treatments reached an appropriate germination level for soybean seeds, showing germination percentages above 80% – minimum value referred for Brazil (2005) – characterizes the absence of harmful effects under this variable at the time of sowing up to three days after seed treatment.
A reduction of seeds'germination percentage can be observed as and when storage period increases after the insecticide treatment (Figure 1A), particularly for carbofuran and acephate on day 7 of storage, which conditioned seeds at germination percentages below 80%. Acephate was the insecticide which showed a greater reduction in seed germination during storage periods, providing a reduction of 3.089 percentage points (pp) in germination for each day of seed storage.

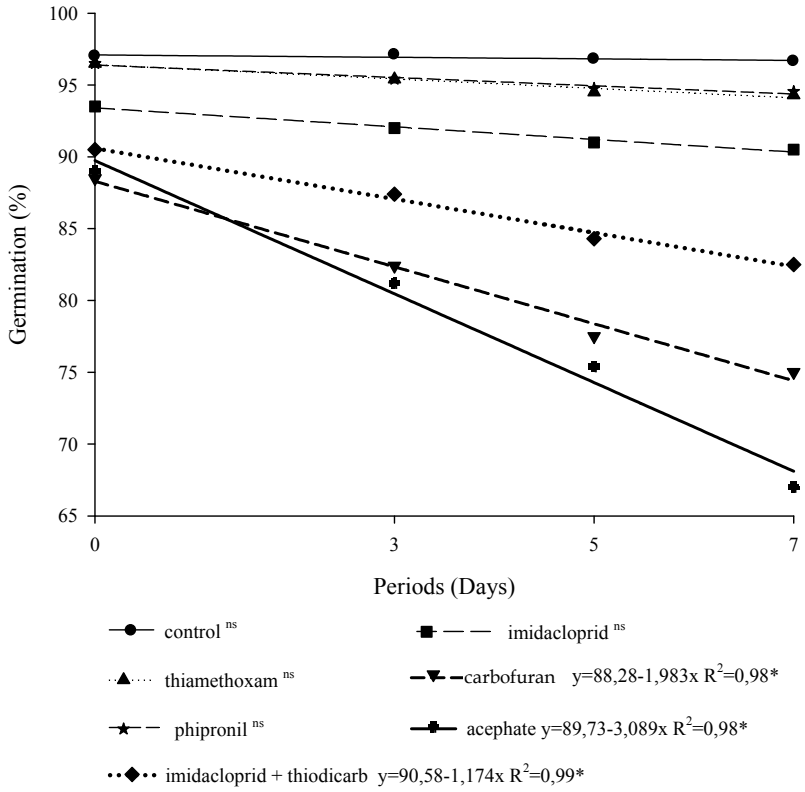


Fig. 1 A. Germination percentage after insecticide treatments in four periods of storage (Dan *et al*., 2011).

Regarding to vigor, determined by speed of emergence index (Figure 2), there was no significant difference between the non-treated witness and phipronil and thiamethoxam treatments, which means that speed of emergence was not affected by the respective treatments when submitted to storage periods. For Horii and Shetty (2007), insecticides such as thiamethoxam may help in the metabolic pentose phosphate pathway, benefiting the hydrolysis of reserves and increasing the availability of energy to the germination process

and seedling emergence. Grisi *et al.* (2009) observed neither vigor nor emergence alteration of sunflower seeds treated with thiamethoxam and phipronil.

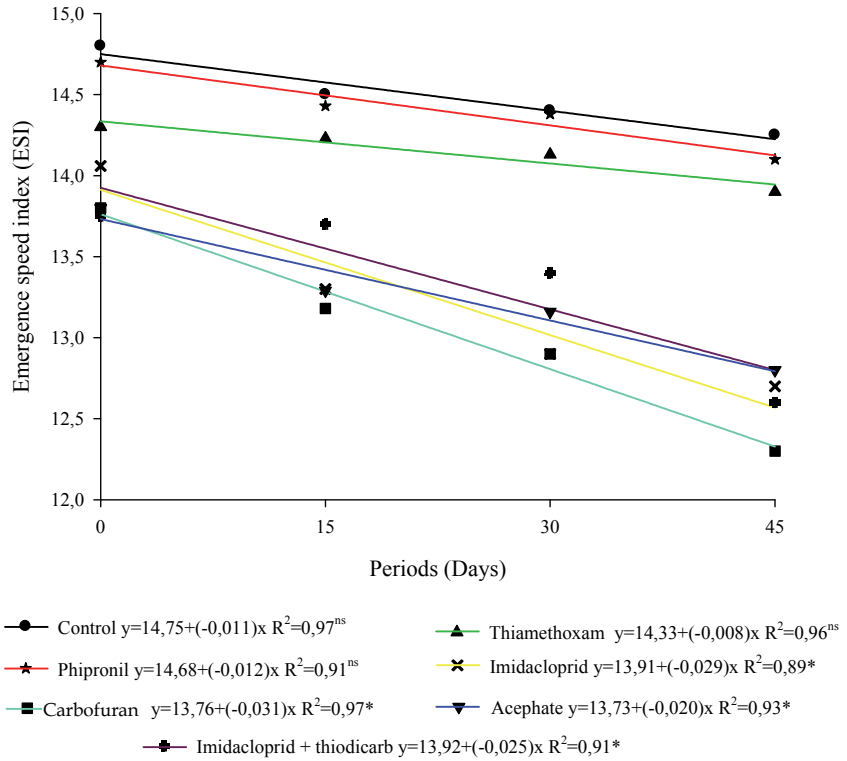


Fig. 2. Speed of emergence index (SEI) of soybean seedlings after insecticide treatments in four periods of storage (Dan *et al*., 2010).

In general, a reduction in speed of emergence index (SEI) can be observed as when storage period increases, particularly enhanced in the treatments with imidacloprid, imidacloprid plus thiodicarb, carbofuran and acephate (Figure 2). Nevertheless, Castro *et al.* (2008) observed higher vigor in soybean seeds treated with imidacloprid. Bittencourt *et al.* (2000) have found no effects of the insecticides thiodicarb, imidacloprid plus thiodicarb in corn seedlings emergence. However, treated seeds were not stored in these studies.

Within seed treatments assessed, the insecticide carbofuran presented a higher reduction in speed of emergence index for each day of storage, reaching 0.031 reduction unit. Godoy *et al.* (1990) observed lower percentages and emergence speed when corn seeds were insecticide treated using carbofuran. During short-term storage Dan *et al.* (2011) observed a reduction of 5.8% in SEI in a seven-day storage.

Results obtained according to seedling primary root length of soybean crops are shown in Figure 3. Except for the witness, all of the other insecticide treatment negatively influenced soybean seedling radicular growth. This fact occurred mainly when treated seeds were stored.

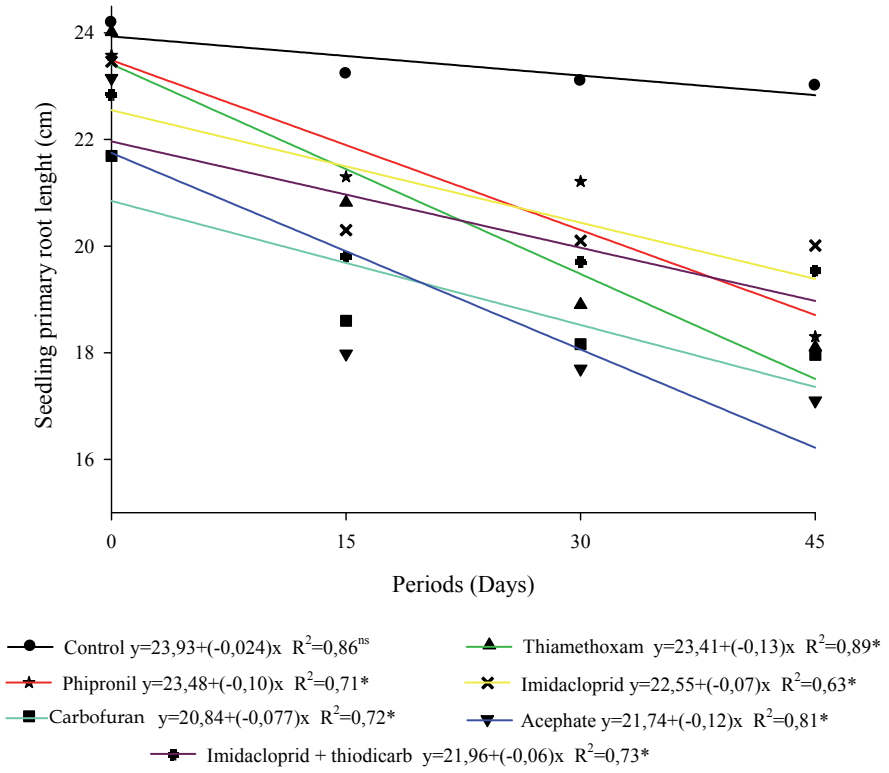


Fig. 3. Soybean seedling primary root length (cm) after insecticide treatment in four periods of storage (Dan *et al*., 2010).

Phipronil, acephate and thiamethoxam treatments presented the highest reductions in primary root length in relation to the increase of storage period, showing reductions reaching to 0.10; 0.12 and 0.13 for each day of storage, respectively. These results are in accordance to those found by Silveira *et al.* (2001), in which the insecticide phipronil produced a phytotoxic effect on corn seedling radicular growth. Similarly, during short-term storage, Dan *et al.* (2011) observed that soybean seed incecticide treatment using acephate presented the highest reduction in the primary root length in relation to storage period, showing reductions reaching 0.667 cm each day. Nevertheless, for thiamethoxam, Nunes (2006) assessed that the effect of this insecticide on seed germination produces plants with greater root enlargement and fasciculation, simultaneously to a higher growth of the aerial part. These facts were not observed in this present study.

Moreover, significant reductions in the dry matter of plants from seeds which were insecticide treated and subsequently stored were observed. According to Dan *et al.* (2011), a reduction in dry matter accumulation from the aerial part of soybean plants was observed when compared to the witness (Figure 4) in the treatments using imidacloprid, imidacloprid plus thiodicarb, acephate and carbofuran. It was observed no significant differences in the

treatments using thiomethoxam and phipronil when compared to the witness in relation to dry matter accumulation from the aerial part of soybean plants.
In the treatment using phipronil for corn seeds, Silva & Silva (2009) found similar stem matter in relation to the witness, thus, supporting the results obtained in this study.
Therefore, it is important to note that treatments using the insecticides acephate and imidacloprid plus thiodicarb presented the highest reduction indexes of dry matter accumulation for each day of storage in the order of 4.919 and 4.777%, respectively. Whereas, the treatment using carbofuran presented the lowest dry matter accumulation in all storage periods when compared to the witness. The deletery effects of this insecticide were observed even under conditions when sowing was accomplished right after seed treatment.

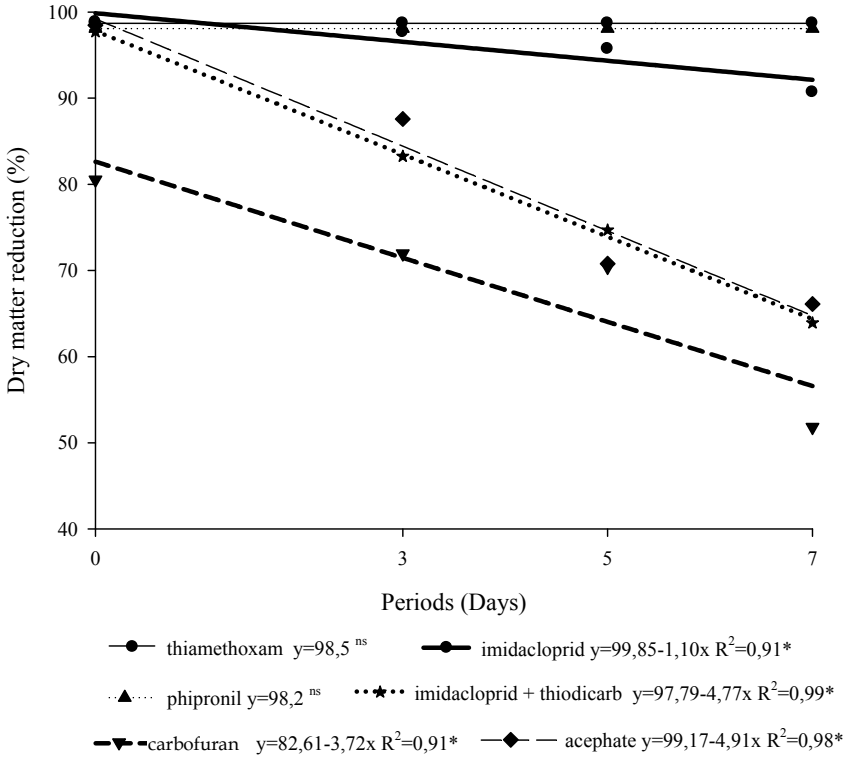


Fig. 4. Dry matter reduction from aerial part of soybean plants (according to the witness) from insecticide treated seeds in four periods of storage (Dan *et al*., 2011).

Negative interferences were verified regarding vigor of soybean seeds treated with insecticides and subsequently stored. According to Dan *et al.* (2010), by assessing normal seedlings' percentage after accelerated aging (Figure 5) in soybean seeds treated with different kinds of insecticides, observed that all treatments reduced seeds' vigor in relation to storage period.

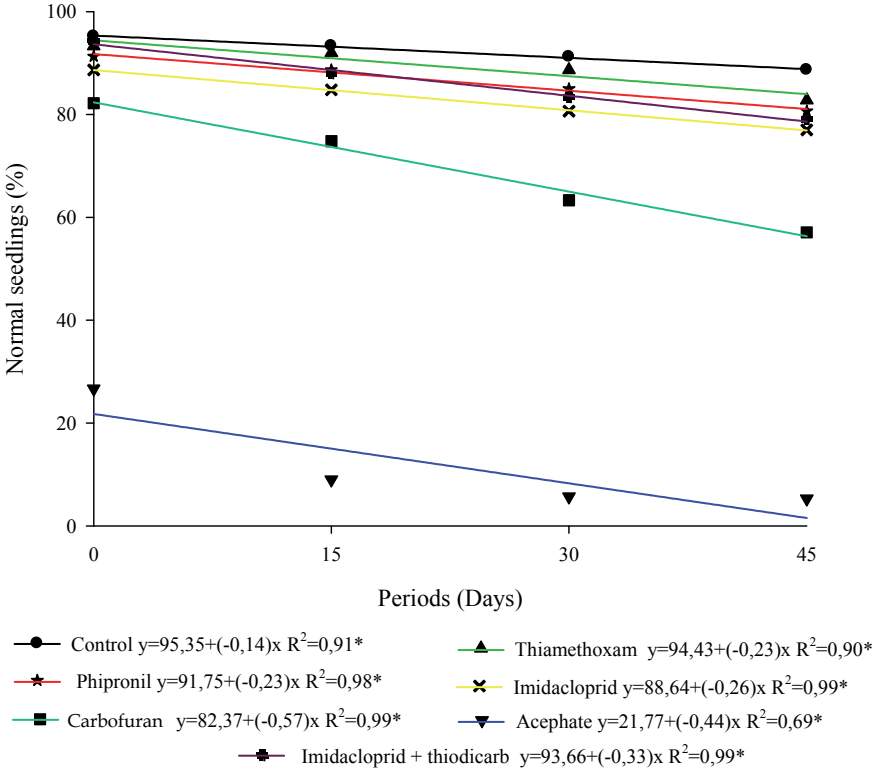


Fig. 5. Normal seedlings (%) obtained from seed accelerated aging test after insecticide treatment in four periods of storage (Dan *et al*., 2010).

At time zero, except for acephate, all treatments showed high vigor level, being over 80%. However, soybean seeds insecticide treated by using acephate obtained only 26.6% of normal seedlings, indicating that this insecticide drastically reduced vigor in soybean seeds (Figure 5).

Decreases in physiological potential of insecticide treated seeds may be related to the formation of free radicals, as a response to exogen stress produced by insecticides from carbamate and organophosphate groups (Soares & Machado, 2007). Free radicals provide oxidative modification of proteins; DNA injuries and cell membrane lipids peroxidation. Many of these reactive oxygen species are formed from xenobiotic metabolism deriving one or more reactive byproducts (Delgado, 2006). Several pesticides, such as organochlorides (endosulfan) and organophosphorates (acephate) have toxic effects involving free radicals. Oxidative stress induction (imbalance between the production and catalization of free radicals) and antioxidant system alteration by pyrethroids have been shown by a number of authors (Braguini, 2005).

According to the progressive increase of days seeds were kept stored after insecticide treatment, reductions in percentage of normal seedlings obtained from accelerated aging

test were also markedly verified by using the insecticide carbofuran, following the same behavior from those results obtained by acephate (Figure 5). However, soybean seeds' treatment by using carbofuran presented a steeper reduction in vigor (0.57 pp) for each storage day. These results are in accordance to Fessel *et al.* (2003) when verifying that seeds' vigor diminished as when storage period of treated seeds increased.

Nevertheless, despite vigor reductions have been occurred according to the increase of storage period, it is possible to verify that soybean seed insecticide treatments using phipronil, thiamethoxam, imidacloprid and imidacloprid plus thiocarb have established a vigor level of 80%. Thus, soybean seeds utilized in these insecticide treatments still presented good quality when submitted to a 45-day storage (Figure 5).

Therefore, Dan *et al*. (2010) concluded that the application of insecticides such as carbofuran and acephate is harmful to physiological quality of cultivar M-SOY 6101 of soybean seeds for a 45-day storage period. In addition, the reduction in physiological quality of conditioned seeds by using the insecticides carbofuran, acephate, imidacloprid and imidacloprid plus thiocarb is intensified according to the extension of treated seed storage period. Therefore, seed insecticide treatment should be carried out next to sowing time.

There have been some reports saying that insecticides not only protect crops from insect attacks but also act as bioactivators, improving crop performance, however, results are contradictory in relation to these effects. Regarding the effects on seed insecticide application, Ceccon *et al*. (2004) observed that the insecticides phipronil and thiamethoxam stood out concerning agronomic parameters, corn harvesting, in areas highly infested by burrowing bugs and white grubs but did not present any increase on grain yield when compared to the witness.

Ester *et al.* (1997) did not observe any phytotoxic effect on the use of insecticides on seeds; however, she verified a reduction in emergence speed. On the other hand, Tavares *et al.* (2007) did not observe any difference in germination and vigor when different doses of thiamethoxam were used in the treatment. These authors did not find any difference in the development of both hypocotyl and primary root of soybean seedlings after the application of five doses of the product.

By assessing the effects on the application of insecticides, fungicides and their associations with corn seed quality, Takahashi & Cícero (1986) observed that the insecticides such as deltametrine 2.5% CE plus piperonyl and avermectim butoxide 0.36% SL were efficient on protecting seeds throughout a 12-month storage period.

Smiderle (1998) concluded that the insecticides deltametrine and chlorpyrifos, isolated or associated, phosphine as well as diatomaceous earth similarly promote control of insect pests which occurs in storage and do not cause toxicity to corn seeds. In addition, physiological quality of corn seeds is preserved by chemical treatments and diatomaceous earth.

The accomplishment of seed treatment at the time prior to sowing is a common practice for many crops such as soybean and corn. Nevertheless, due to adverse climatic conditions, treated seeds are stored for a short-term period for further utilization until those conditions become favorable to sowing. Generally, storage environment also does not meet the ideal storage conditions, in other words, treated seeds are stored in natural environments eventually exposed to high temperatures and moisture, which may jeopardize their physiological performance (Dan *et al*., 2011).

It is important to highlight that the function of storage is to preserve the initial quality of products, avoiding its deterioration. Thus, the objective of storage is to maintain a good

level of seed quality until they are commercialized and/or used for sowing. Seeds storage period in an environment is determined by genetic inheritance and level of seed deterioration at the beginning of storage (Fontes & Mantovani, 1993; Kelly & George, 1998). Furthermore, Machado (2000) reported the dependence on chemical treatment effectiveness due to a number of factors, among them, the vigor of seeds at the time of the product application.

4. Perspectives and final considerations

In general, although there are some variations on results in some studies concerning the influence of insecticide treatment on physiological quality of seeds from different crops, it can be inferred that insecticides application is harmful to the physiological quality of soybean seeds during long storage periods. The intensity of these negative effects on seeds is closely related to initial vigor before storage. In lower vigor lots, depending on the insecticide used in the treatment, reduction of physiological quality can cripple commercialization in a short-term storage period.

However, regardless of species, the reduction in the physiological quality of seeds conditioned by insecticides is enhanced by extending storage period for treated seeds. Therefore, seed insecticide treatment should be accomplished as closer as possible to sowing time.

It is important to note that, other products have been used in seed treatment as fungicides, bioregulators and some micronutrients; therefore, it is assumed that there may be some interaction with insecticide treatment, consequently influencing on the physiological quality of seeds. More information should be provided in order to find more about this interaction as well as the most appropriate time to treat the seeds.

5. References

BAUDET, L.; PESKE, T. S. A logística do tratamento de sementes. Seed News, Pelotas, n. 1, p. 22-25, 2006.

BARBOSA, F.R.; SIQUEIRA, K.M.M.; SOUZA, E.A.; MOREIRA, W.A.; HAJI, F.N.P.; ALENCAR, J.A. Efeito do controle químico da mosca-branca na incidência do vírus-do-mosaico-dourado e na produtividade do feijoeiro. Pesquisa Agropecuária Brasileira, Brasília, v.37, n.1, p.879-883, 2002.

BARROS, R. G.; YOKOYAMA, M.; COSTA, J. L. da S. Compatibilidade do inseticida thiamethoxan com fungicidas utilizados no tratamento de sementes de feijoeiro. Pesquisa Agropecuária Tropical, Goiânia, v. 31, p. 153-157, 2001.

BARROS, R.G.; BARRIGOSSI, J.A.F.; COSTA, J.L.S. Efeito do armazenamento na compatibilidade de fungicidas e inseticidas, associados ou não a um polímero no tratamento de sementes de feijão. Bragantia, Campinas, v.64, n.3, p.459-465, 2005.

BITTENCOURT, S. R. M; FERNANDES, M. A; RIBEIRO, M. C; VIEIRA, R. D. Desempenho de sementes de milho tratadas com inseticidas sistêmicos. Revista Brasileira de Sementes. v.22, n.2, p. 86-93, 2000.

BRAGUINI, W. L. Efeitos da Deltametrina e do Glifosato, sobre parâmetros do metabolismo energético mitocondrial, sobre membranas artificiais e naturais e experimentos *in vivo*. Curitiba: UFP, 2005.

BRASIL. Instrução Normativa n.25, de 16 de dezembro de 2005. Diário Oficial da República Federativa do Brasil, Poder Executivo, Brasília, DF, 16 dez. 2005. p.18.

CARVALHO, N. M. et al. Aplicação pré-colheita de dessecante de soja (*Glycine max* (L) Merrill) cultivar Viçoja: II-Efeitos imediatos sobre a germinação das sementes. Científica, Jaboticabal, v. 6, n. 2, p. 209-213, 1978.

CARVALHO, N.M.; NAKAGAWA, J. Sementes: ciencia, tecnologia e producao. 3. ed., Campinas: Fundacao Cargill, 1988. 424p.

CASTRO, N.R.A. Sorção, degradação e lixiviação do inseticida Thiamethoxam em latossolo e argissolo. Dissertação de mestrado. Lavras, MG, UFLA. 173 p. 2005.

CASTRO, G. S. A. et al. Tratamento de sementes de soja com inseticidas e um bioestimulante. Pesquisa Agropecuária Brasileira, Brasília, DF, v. 43, n. 10, p. 1311-13188, 2008.

CECCON, G. et al. Efeito de inseticidas na semeadura sobre pragas iniciais e produtividade de milho safrinha em plantio direto. Bragantia, Campinas, v. 63, p. 227-237, 2004.

DAN, L. G. M.; DAN, H. A.; BARROSO, A. L. L.; BRACCINI, A. L. Qualidade fisiológica de sementes de soja tratadas com inseticidas sob efeito do armazenamento. Revista Brasileira de Sementes, Londrina, v. 32, n. 2, p. 131-139, 2010.

DAN, L. G. M.; DAN, H. A.; BRACCINI, A. L.; ALBRECHT, L. P.; RICCI, T. T.; PICCININ, G. G. Desempenho de sementes de soja tratadas com inseticidas e submetidas a diferentes períodos de armazenamento. Revista Brasileira de Ciências Agrárias. Recife, v. 6, n. 2, p. 215-222, 2011.

DELOUCHE, J.C. Seed quality and storage of soybeans. Soybean production, protection and utilization. Urbana – Champaign, University of Illinois International Agricultural Publications, Intsoy, n. 6, p.86-107, 1975.

DELGADO, E.H.B. Disfunção respiratória mitocondrial e estresse oxidativo após exposição crônica ao malathion. Dissertação (Mestrado) - Universidade do Extremo Sul Catarinense, Criciúma, 2006 .

EMBRAPA. Tecnologias de produção de soja. Embrapa: Sistemas de Produção. Brasília, 2002. 190 p.

ESTER, A.; VOGEL, R. de; BOUMA, E. Controlling *Thrips tabaci* (Lind.) in leek by film-coating seeds with insecticides. Crop Protection, Great Britain, V. 16 n. 7, p. 673-677, 1997.

FERREIRA, A.G.; BORGHETTI, F. (Org.) Germinação: do básico ao aplicado. Porto Alegre: Artmed. 2004. 323 p.

FESSEL, S. A.; MENDONCA, E. A. F.; CARVALHO, R. V. Effect of chemical treatment on corn seeds conservation during storage. Revista Brasileira de Sementes, Pelotas, v. 25, n. 1, p. 25-28, 2003.

FONTES, R.A.; MANTOVANI, B.H.M. Armazenamento de sementes. In: EMBRAPA. Centro Nacional de Pesquisa de Milho e Sorgo. Tecnologia para produção de sementes de milho. Sete Lagoas, 1993. p.49-61. (EMBRAPA/CNPMS/Circular técnica, 19).

FOSSATI, M. L. Influências do tratamento de sementes de soja com inoculante, micronutrientes e fungicidas sobre a população inicial de plantas, nodulação, qualidade de sementes e rendimento de grãos. 2004. 23 f. Dissertação (Mestrado em Fitotecnia)- Universidade Federal de Pelotas, Pelotas, 2004.

GASSEN, D. N. Manejo de pragas associadas à cultura do milho. Passo Fundo: Aldeia Norte, 1996. 134p.

GIANASI, L. et al. Eficiência do fungicida captan associado a outros fungicidas no tratamento químico de sementes de soja. Summa Phytopathologica, Jaboticabal, v. 26, n. 2, p. 241-245, 2000.

GODOY, J.R.; CROCOMO, W.B.; NAKAGAWA, J.; WILCKEN, C.F. Efeito do armazenamento sobre a qualidade fisiológica de sementes tratadas com inseticidas sistêmicos. Científica, Jaboticabal, v.18, n.1, p.81-93, 1990.

GOULART, A. C. P.; FIALHO, W. F. B.; FUJINO, M. T. Viabilidade técnica do tratamento de sementes de soja com fungicidas antes do armazenamento. Dourados: Embrapa Agropecuária Oeste, 1999. 41 p. (Boletim de Pesquisa e Desenvolvimento, 2).

GOULART, A. C. P.; PAIVA, F. de A.; ANDRADE, P. J. M. Controle de fungos em sementes de soja (*Glycine max*) pelo tratamento com fungicidas. Summa Phytopatologica, Jaguariúna, v. 21, n. 3/4, p. 239-244, 1995.

GOTARDO, M; BITTENCOURT, S. R. M; PEREIRA, L. M. A; VIEIRA, R. D; GOTARDO JÚNIOR, J. R. Qualidade fisiológica de sementes de milho tratadas com diferentes inseticidas. Revista Ceres, Viçosa, v.48, n.278, p. 511-516, 2001.

GUIMARÃES, R. N. et al. Efeito do tratamento de sementes com inseticidas na emergência e altura de plântulas de feijão. In: CONGRESSO NACIONAL DE PESQUISA DE FEIJÃO, 8., 2005, Goiânia. Anais... Goiânia: Embrapa Arroz e Feijão, 2005. p. 94-99. (Documentos, 182).

GRISI, P. U.; SANTOS, C.M.; FERNANDES, J. J.; SÁ JÚNIOR, A. Qualidade das sementes de girassol tratadas com inseticidas e fungicidas. Bioscience Journal, Uberlândia, v.25, n.4, p.28-36, 2009.

HORII, P. M.; K. SHETTY. Enhancement of seed vigour following insecticide and phenolic elicitor treatment. Bioresource Technology, v.98, p.623-632, 2007.

KASHYPA, R. K.; CHAUDHARY, O. P.; SHEORAN, I. S. Effects of insecticide seed treatments on seed viability and vigour in wheat cultivars. Seed science and Technology, Zurich, v. 22, n. 3, p. 503-517, 1994.

KELLY, A.F.; GEORGE, R.A.T. Encyclopedia of Seed Production of World Crops. 1998. p.125-137.

LIMA, A.C. Qualidade na producao de sementes atraves do sistema integrado de gestao. Anuário Abrasem, 2003. p. 112-124.

LIMA, R.M. Efeito do tamanho das sementes sobre alguns atributos fisiologicos e agronomicos. Anuário Abrasem, Brasilia, p. 168, 1997.

MACHADO, J. C. Patologia de sementes: significado e atribuições. In: CARVALHO, N. M.; NAKAGAWA, J. (Eds.). Sementes: ciência, tecnologia e produção. 4. ed. Jaboticabal: FUNEP, 2000. p. 522-588.

MARCOS FILHO, J. Teste de envelhecimento acelerado. In: In: KRZYZANOWSKI, F.C.; VIEIRA, R.D.; FRANCA NETO, J. de B. (Eds.). Vigor de sementes: conceitos e testes. Londrina: ABRATES. 1999. Cap.3. p.1-24.

MAPA. Ministério da Agricultura, pecuária e abastecimento. Brasília. Disponível em: <http://mapa.gov.br>. Acesso em novembro de 2009.

MENTEN, J. O. M. Tratamento de sementes com inseticidas. In: SEMANA DE ATUALIZAÇÃO EM PATOLOGIA DE SEMENTES, 2., 1991, Piracicaba. Anais... Piracicaba: ESALQ/USP, 1991. p. 278-279.

MENTEN, J. O. M. Tratamento químico de sementes. In: SIMPÓSIO BRASILEIRO DE PATOLOGIA DE SEMENTES, 4., 1996, Gramado. Anais... Campinas: Fundação Cargill, 1996. p. 3-23.

NOVEMBRE, A. D. L. C.; MARCOS FILHO, J. Tratamento fungicida e conservação de sementes de feijão. Revista Brasileira de Sementes, Brasília, DF, v. 13, n. 2, p. 105-113, 1991.

NUNES. J.C., Bioativador de plantas, Seeds News. Pelotas, v.3, n.5, p.30-31, 2006.

OLIVEIRA, L. J.; CRUZ, I. Efeito de diferentes inseticidas e dosagens na germinação de sementes de milho (*Zea mays* L.). Pesquisa Agropecuária Brasileira, Brasília, DF, v. 21, n. 6, p. 578-585, 1986.

PADILHA, L.; FARONI, L.R.D.A. Importância e forma de controle de *Rhizopertha dominica* em grãos armazenados. In: SIMPÓSIO DE PROTEÇÃO DE GRÃOS ARMAZENADOS. Passo Fundo, 1993. Anais. Passo Fundo: EMBRAPA, CNPT, 1993. p.52-58.

PEREIRA, C. E. Peliculização e tratamento fungicida de sementes de soja: efeitos no armazenamento e na inoculação com *Bradyrhizobium*. 2005. 114 f. Dissertação (Mestrado em Fitotecnia)-Universidade Federal de Lavras, Lavras, 2005.

PESKE, S.T.; BAUDETT,L.M.; ROSENTHAL, M.D.; ROTA, G.R.M. Sementes: fundamentos cientificos e tecnologicos. Pelotas: Universidade Federal de Pelotas. Ed. Universitaria, p. 205-352, 2003.

POPINIGIS, F. Fisiologia da semente. 2. ed. Brasília: Agiplan, 1985. 289p.

ROCHA JUNIOR, L.S. Qualidade física e fisiológica de sementes de soja (*Glycine Max* (L.) Merill), cultivar IAC-17, em função da colheita, tamanho da semente e da armazenagem. Campinas: Unicamp, 1999. 59f. Mestrado em Engenharia Agricola - Faculdade de Engenharia Agricola da Unicamp.

RESENDE, P.M. de; MACHADO, J.D.C.; GRIS, C.F.; GOMES, L.L.; BOTREL, E.P. Efeito da semeadura a seco e tratamento de sementes, na emergencia, rendimento de graos e outras caracteristicas da soja (*Glycine Max* (L.) Merrill). Ciência e Agrotecnologia, Lavras, v. 27, n. 1, p. 76-83, 2003.

SANTOS, J. P.; MANTOVANI, E. C. Perdas de grãos na cultura do milho; pré-colheita, colheita, transporte e armazenamento. Sete Lagoas, EMBRAPA-CNPMS, 1997. 40p. (EMBRAPACNPMS. Circular Técnica, 24).

SILVA, M. T. B. Inseticidas na proteção de sementes e plantas. Seed News, Pelotas, v. 2, n. 5, p. 26-27, 1998.

SILVA, M.M.; SILVA, T.R.B. Qualidade fisiológica de sementes de milho em função do tratamento com inseticidas. Cultivando o Saber, Cascavel, v.2, n.1, p.91-98, 2009.

SILVEIRA, R. E.; MACCARI, M.; MARQUEZI, C. F. Avaliação do efeito de inseticidas aplicados via tratamento de sementes sobre o desenvolvimento de raízes de milho, na proteção de pragas do solo. In: REUNIÃO SUL-BRASILEIRA SOBRE PRAGAS DE SOLO, 8., 2001,Londrina. Anais. Londrina: Embrapa Soja, 2001. p. 246-249.

SMIDERLE, O. J.; CÍCERO, S. M. Tratamento inseticida e qualidade de sementes de milho. Revista Brasileira de Sementes, Brasília, DF, v. 20, n. 2, p. 462-469, 1998.

SOARES, A. M. S.; MACHADO, O. L. T. Defesa de plantas: Sinalização química e espécies reativas de oxigênio. Revista Trópica – Ciências Agrárias e Biológicas. v.1, n.1, p.9-19, 2007.

TAKAHASHI, L.S.A.; CICERO, S.M. Efeito da aplicação de inseticidas e fungicidas e suas associações na qualidade de sementes de milho. Revista Brasileira de Sementes, v.8, n.1, p.85-100, 1986.

TAVARES, S.; CASTRO, P.R.C.; RIBEIRO, R.V.; ARAMAKI, P.H. Avaliação dos efeitos fisiológicos de thiametoxan no tratamento de sementes de soja. Revista de Agricultura, Piracicaba, v.82, n.1, p.47-54, 2007.

YAMADA, H. Pré and post-harvest control for the production of quality seed of vegetable. In: Textbook Vegetable Seed, Tsukuba. Japan, 1989. 133p.

ZAMBOLIM, L. Sementes: qualidade fitossanitária. Viçosa: UFV, 2005. 502 p.

15

The Effect of Insecticides on Pest Control and Productivity of Winter and Spring Oilseed Rape (*Brassica napus* L.)

Eglė Petraitienė, Irena Brazauskienė and Birutė Vaitelytė
Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry
Lithuania

1. Introduction

Oilseed rape (*Brassica napus* L.) is an important crop for food industry, bio-fuel industry and as a rotation plant in many countries (Williams, 2010). In Lithuania, oilseed rape is also one of the most promising crops. According to Lithuanian department of statistics, at the end of 2010, oilseed rape was cultivated on an area of 255 957 ha, of which spring rape accounted for 63.9% (163 600 ha) (Lithuanian Department of Statistics).
Various pests attack oilseed rape at different time and damage different plant parts (Williams, 2004). In Europe, chemical insecticides are usually used for pest management in oilseed rape crops and application of insecticides is still an integral part of insect pest management in this crop (Cook et al., 2004). Insecticides are used routinely and prophylactically independent of pest incidence. Insecticides are applied in oilseed rape crop several times during the plant vegetation. One of the most important targets for applying insecticides is a substantial seed yield increase (Williams, 2004).

1.1 Pollen beetle, *Meligethes aeneus* (Coleoptera, Nitidulidae)

Meligethes aeneus is one of the most important pests in Europe visiting oilseed rape. Pollen beetle damages flower buds and can cause significant seed yield losses (Ruther & Thiemann, 1997). Both adults and larvae can be harmful (Blight & Smart, 1999). Sometimes yield losses are of up to 50% (Kirch, 2006). In the United Kingdom, damage caused by pollen beetle was in the range 15.8 - 20.5% (Cook et al., 2004). In Germany, losses caused by *M. aeneus* in oilseed rape were from 20% to 100% (Heimbach et al., 2007) and in Sweden 70% (Kazachkova, 2007). Pollen beetle can cause serious yield losses, and for spring oilseed rape more than 80% yield reduction can occur (Hansen, 2003a; 2003b). Pests cause visible direct losses in seed yield and decrease strength of plant response to favourable environmental and agricultural conditions. The beetles are controlled with different insecticides, but mainly with pyrethroids. It was reported that *M. aeneus* is an example of insect species that can develop strong resistance mechanisms to most active ingredients used to control it in Europe (Hansen, 2003a; Wegorek, 2005; Heimbach, 2007).
Over the past few years, resistance of *M. aeneus* to pyrethroids has increased in Europe (Germany, Denmark, Switzerland and Sweden) (Hansen, 2003a; Derron et al., 2004; Heimbach et al., 2007; Kazachkova, 2007). In Poland, *M. aeneus* showed a high tolerance to

beta – cyfluthrin, also tau-fluvalinate and neonicotinoid resistance were recorded, but just in some treatments, therefore several insecticide treatments are often necessary against pollen beetle (Wegorek et al., 2009).
The results from Denmark show that no insecticide resistance to the organophosphate fenitrothion has developed among the *M. aeneus*. However, a relatively high insecticide resistance to lambda-cyhalothrin and to a lesser extent to tau-fluvalinate was recorded. The most common insecticides used against *M. aeneus* are the pyrethroids and especially lambda-cyhalothrin, which have been used for many years. Tau-fluvalinate has been used very little against *M. aeneus* but over the last two years the use has been increasing. However, insecticide resistance has developed against tau-fluvalinate and this could be due to cross-resistance (Decoin, 2002). It has been concluded that many Danish populations of *M. aeneus* are resistant to the pyrethroid lambda-cyhalothrin and to a lesser extent to the pyrethroid tau-fluvalinate. However, no resistance to the organophosphate fenitrothion was found (Hansen, 2008).
In Estonia, the impact of minimized and standard cropping systems on the abundance of old and new generations of *M. aeneus* was assessed in spring oil seed rape. Insecticide treatments were done at or above the *M. aeneus* threshold level. Treatments reduced the size of the new generation, but not significantly, because the maximum numbers of new generation adults emerge from the soil from much lower old generation beetle densities. Application of insecticide not only results in more buds and flowers for feeding and egg-laying by *M. aeneus* but probably also kills its natural enemies, which have potential to decrease the size of the new generation of *M. aeneus* (Veromann et al., 2008).
In Lithuania, the findings of pest abundance assessments indicate that *M. aeneus* tended to occur in spring rape during the stem elongation – budding stages. The population of pollen beetle was found to be on the increase during the experimental period and the efficacy of the insecticides tested tended to decline. A significant yield reduction ranging from 3.3 to 30.1%, resulting from the damage of pollen beetle, was identified (Petraitiene et al., 2008).

1.2 Cabbage stem weevil, *Ceutorhynchus pallidactylus* (syn. *C. quadridens*) (Coleoptera, Curculionidae)

C. pallidactylus is one of the most important stem-mining pests. Cabbage stem weevil is widely distributed on oilseed rape crops in Central and Northern Europe (Eickermann, 2008). This pest attacks oilseed rape stems and can cause significant yield losses (Dechert & Ulber, 2004). Largest damages are caused by larvae of *C. pallidactylus,* they usually infest the lateral shoots of the oilseed rape (Barari et al., 2005). Later on larvae are tunnelling their way into the mid-rib of the leaf or the stem. Yield losses caused by *C. pallidactylus* larvae were recorded up to 50% in the United Kingdom (Alford et al., 2003) and 20% in Germany (Lanschreiber, 2005).
Synthetic pyrethroids are the predominant insecticides applied on oilseed rape. However, this group of insecticides is non-selective for non-target insects (Williams 2004). Further frequent usage of pyrethroids can result in insecticide resistance (Hansen, 2003a). Plant architecture and development can influence pest abundance (Büchs & Katzur, 2004; Williams, 2004). Infestation by *C. pallidactylus* increases as plant density decreases because a higher number of leaves and larger leaf size at low plant density increases oviposition (Nuss & Ulber, 2007). Generally, hybrid cultivars are considered to have higher compensation ability to pest damage by growing more vigorously (Lamb, 1989). Classical crop breeding

can play an important role for developing oilseed rape cultivars being resistant to insect pests, which could reduce the need for insecticide application (Williams, 2004).

1.3 Cabbage seedpod weevil, *Ceutorhynchus obstrictus* (syn. *C. assimilis*) (Coleoptera: Curculionidae)

C. obstrictus is an important pest of flowering period causing yield losses (Carcamo et al., 2009). Oilseed rape growers in North America have significant seed yield reductions (15–35%) due to *C. obstrictus* (Buntin, 1999a). Cabbage seedpod weevil is more damaging to winter rape than to spring rape (Dosdall, 2009). Feeding by *C. obstrictus* larvae can cause yield losses of 19-80% (autorius). In Europe, this pest of oilseed rape reduces yield of infected pods by about 18% (Alford et al., 2003; Wiliams, 2004; Cook et al., 2006).

Neonicotinoid insecticides have been used for several years in oilseed rape as seed treatments for reducing damage of *C. obstrictus*. The neonicotinoids clothianidin and imidacloprid were investigated to determine their effects on preimaginal development and on emergence of new-generation adults of *C. obstrictus* in comparison with effects of lindane, a chlorinated hydrocarbon seed treatment. Mean numbers of second- and third-instar larvae were significantly higher in plants seed-treated with lindane than in plants treated with the neonicotinoid compounds, even though weevil oviposition was similar for all treatments. Emergence of new-generation adults was reduced by 52 and 39% for plants seed-treated with clothianidin and imidacloprid, respectively, compared with emergence from plants treated with lindane. Seed treatment with both clothianidin and imidacloprid produced systemic insecticidal effects on larvae of *C. obstrictus*, with clothianidin slightly more effective than imidacloprid. It has been concluded that use of clothianidin or imidacloprid as seed treatments can comprise an important component in the integrated management of *C. obstrictus* in oilseed rape (Dosdall, 2009).

Experiments examining the efficacy, timing and number of applications of various insecticides were used to assess the relationship between *C. obstrictus* pod infestation and yield loss in winter oilseed rape. The pyrethroid insecticides (permethrin, esfenvalerate, bifenthrin, and zeta-cypermethrin) were not significantly different in efficacy in any trial and were more effective in reducing *C. obstrictus* infestations than the organophosphate insecticides (Buntin, 1999b). Two insecticide applications during flowering were usually needed to effectively reduce the number of adults and to prevent seed injury. Larval injury primarily affected grain weight but did not consistently affect kernel weight or grain oil content. Yield loss increased linearly by about 1.7% for each 1% increase in percentage of infested pods, when larval infestation of pods exceeded 22% infested pods. These results support findings from Europe that canola can tolerate pod infestations of <26% without measurable yield loss (Lerin, 1984). Economic injury levels for varying control costs and commodity values ranged from 26 to 40% infested pods (Buntin, 1999b).

In Europe, pyrethroid insecticides including deltamethrin and alpha-cypermethrin were used to control *C. obstrictus* (Garthwaite et al., 1995). Pyrethroid insecticides were applied during flowering to kill adults before oviposition occurs and have less adverse impact on *C. obstrictus* parasitoids and canola pollinators than organophosphate insecticides (Murchie et al. 1997).

1.4 Brassica pod midge, *Dasineura brassicae* (Diptera: Cecidomyiidae)

D. brassicae is a serious pest of oilseed rape in many countries of Europe. It is one of the pests that infests oilseed rape at flowering and pod setting stages which is considered the most

suitable time for egg-laying (Murchie et al., 1997). As much as 82% of seed weight can be lost from *D. brassicae* infested pods (Williams, 2010).
The *D. brassicae* has three generations per year in Europe, two generations of *D. brassicae* can develop in winter oilseed rape (Kirch, 2006). In Czech, pesticide manufacturers recommend applying insecticides at the brassica pod midge flying activity or according to the oilseed rape growth stages during the period from first petals visible ("yellow bud") (GS 59) to full flowering stage (GS 65). Insecticide use against the brassica pod midge in the final stage of flowering (GS 67-68) shows significant yield increases. Researchers found that the best term of treatment pyrethroid Karate Zeon was at the final stage of flowering or according to catches in insect traps that signal the beginning of flight activity of the second generation. The damage done by the second generation seems to be very important economically (Pavela et al., 2007).
The study from Serbia showed that the incidence of brassica pod midge is still relatively low in this country. Brassica pod midge larvae were found in 0.95% of the pods in 2009, with 0.13 larvae per pod. In 2010, the pest was more numerous (0.61 larvae/pod) and its incidence was greater (4.7%) (Milovac et al., 2011).
The biological efficiency of botanical insecticide Nemm (azadirachtin) was compared with the efficiency of some synthetic insecticides (Pavela et al., 2009). It was ascertained that botanical insecticide was very efficient in decreasing the number of damaged oilseed rape pods (ranging from 56.5 to 85.9% compared to untreated plants) and its efficiency was comparable with synthetic insecticides based on Chloronicotinyl (thiacloprid) and Neonicotinoid (acetamiprid). Biological insecticide efficiency was, in some years, even significantly higher compared to pyrethroid (lamda-cyhalothrin). The yield increase resulting from azadirachtin application ranged between 9.3 and 19.4% compared to the control (Pavela et al., 2009).
In recent years, increasing areas of oilseed rape in Lithuania, spread stems and pods damage pests. It seems that the influence of stem and pod pests' damage has changed significantly and probably crossed the economic threshold.
The aim of our study was to present the data from the experiments on insecticides efficacy against pollen beetle (*M. aeneus*), cabbage stem weevil (*C. pallidactylus)* and pod pests (*C. assimilis*, *D. brassicae*) either their effect on the productivity of spring and winter oilseed rape.

2. Materials and methods

Ten field experiments were carried out from 2005 to 2009 at the Institute of Agriculture, Research Centre for Agriculture and Forestry (55° 24′33″ N, 23° 52′00″ E). Six field experiments were carried out in the spring oilseed rape and four experiments were performed in winter oilseed rape crops. Two commonly grown cultivars: spring oilseed rape cultivar Landmark and winter oilseed rape cultivar Libea were used in the experiments. The largest area is sown with these oilseed rape cultivars and the highest yields are produced in the central part of Lithuania. At the experimental site, the mean annual precipitation rate is about 700 mm and the mean daily temperature during the period of M. aeneus damage in May is 12.3°C, in June 15.6°C, and in July 17.7°C. The weather data are taken from the Dotnuva weather station, located at 1 km distance from the experimental fields.
The winter and spring oilseed rape crops were cultivated according to the conventional technology. All of the experiment trials were carried out in a randomized complete block

design with four replicates. Each treatment plot size was 25 m^2 x 4 replicates. For assessing the winter and spring oilseed rape growth stages (GS), the scale described by Lancashire (Lancashire et al., 1991) was used. Insecticides of different chemical classes were used against pollen beetles (*M. aeneus*) taking into account the economic threshold of harmfulness (1-2 beetles per plant). Table 1 presents the characteristics of insecticides used in the experiments. The tested insecticides were applied due to the incidence of pollen beetle usually at inflorescence emergence stage (GS 50-59) in the experiments of winter and spring oilseed rape. All experiments had a control treatment with no insecticides. The insecticides were sprayed with a Hardi trial sprayer at 400 l ha^{-1} spray solution, boom length 2.5 m, nozzle type IDK120 01 and the spraying pressure 3.0 bars per nozzle.

Insecticide	Active ingredients	Chemical class	Dose rates l, kg ha^{-1}
Decis 50 EW	deltamethrin 50 g l^{-1}	pyrethroid	0.125 and 0.15
Fastac EC	alpha-cypermethrin 100 g l^{-1}	pyrethroid	0.15
Bulldock 025 EC	beta-cyfluthrin 25 g l^{-1}	pyrethroid	0.225 and 0.3
Mavrik 2 F	tau-fluvalinate 240 g l^{-1}	pyrethroid	0.3
Proteus 110 OD	deltamethrin+thiacloprid 10+100 g l^{-1}	neonicotinoid + pyrethroid	0.6 and 0.75
Pyrinex Supreme	beta-cyfluthrin+chlorpyrifos 12+250 g l^{-1}	organophosphorus + pyrethroid	0.75, 1.0 and 1.25
Steward EC	indoxacarb 300 g l^{-1}	oxidiazines	0.0425, 0.0625 and 0.085

Table 1. Insecticides, used in the experiments.

In all trial plots, pollen beetle was counted 1, 4 and 7 days and in some cases 10 and 14 days after insecticide application. The assessments of pest abundance (counts of pollen beetles per plant) were done on ten successive plants in three chosen places per each plot.

At fruit development stage (GS 71-73) samples of winter and spring oilseed rape pods were analyzed. At least 200 pods per each plot were examined for presence of larvae. The number of pods infested with cabbage seedpod weevil (*C. obstrictus*) larvae and those infested with brassica pod midge (*D. brassicae*) larvae were estimated, also larvae of cabbage seedpod weevil and brassica pod midge were counted and average number of larvae per assessed pod was estimated.

Stem samples at fruit development stage (GS 73) were analyzed for the presence of cabbage stem weevil (*C. pallidactylus*) larvae damage and the number of larvae exit holes per stem. In each plot, twenty plants were selected at random. In the field, the plants were cut open in the stem area. The presence of larvae damage also and any exit holes were assessed.

The winter and spring oilseed rape seed yield from each plot was harvested separately by a Winterstieger Classic harvester. Seed yield kg ha^{-1} was calculated and adjusted to 9% moisture content. Thousand seed weight (TSW) g was measured in the laboratory by a seed counter Contador and balance Explorer Ohaus.

The experimental data were analyzed separately for each year. Biological efficacy of insecticides against pollen beetle was calculated using Henderson-Tilton's formula (Henderson and Tilton, 1955). All data were analysed using an analysis of variance (ANOVA). The experimental data were log(X+1) or arcsin $\sqrt{X\%}$ transformed before analysis. The least significant difference (LSD) was calculated for P≤0.05, P≤0.01 levels. Analysis of variance was performed using the programme Statistica, 5.5 version.

3. Results and discussion

3.1 The efficacy of insecticides in the control of pollen beetle in spring and winter oilseed rape

M. aeneus was a very common and harmful pest in winter and spring rape crops every year. The date of arrival of first *M. aeneus* beetles to the rape field depended on the air temperature. The first beetles were usually detected in winter or spring rape crops at stem elongation stage (GS 30-39), but their amount reached spray threshold (1-2 beetles/plant) even later – at the inflorescence emergence growth stage (GS 50-53), except for 2008 when in spring oilseed rape their abundance reached threshold even earlier at the very end of stem elongation growth stage (GS 39) (Tables 2, 4). Pollen beetle was more abundant in spring rape and this is in agreement with the findings of other researchers (Alford et al., 2003). Depending on the year, one or two spray applications of insecticides were used to control *M. aeneus*. All insecticides used in the trials were effective against *M. aeneus* and significantly reduced the amount of pollen beetle in sprayed plots, compared with the unsprayed control plots (Tables 2-6). Insecticides Proteus 110 OD (a.i. deltamethrin+thiacloprid 10+100 g l^{-1}) 0.6 and 0.75 l ha^{-1}, Decis 50 EW (a.i. deltamethrin 50 g l^{-1}) 0.125 and 0.15 l ha^{-1} and Fastac EC (a.i. alpha-cypermethrin 100 g l^{-1}) 0.15 l ha^{-1} effectively controlled pollen beetle in spring oilseed rape for 10 days in 2005 (I spray application) and in 2006 (Table 2).

Higher doses of Proteus and Decis were more effective, compared with lower doses of these insecticides; however the differences were not significant. There were no significant differences in the efficacy against pollen beetle of pyrethroids (deltamethrin and alpha-cypermethrin) and neonicotinoid thiacloprid in 2005 and 2006 cropping seasons.

Similar results were obtained in 2007 and 2008 cropping seasons in spring and winter oilseed rape, where three dose rates of organophosphorus insecticide Pyrinex Supreme (a.i. beta-cyfluthrin+chlorpyrifos 12+250 g l^{-1}) were included into the trial (Tables 3 and 4). Efficacy of insecticide Pyrinex Supreme was compared with Buldock (a.i. beta-cyfluthrin 25 g l^{-1}) and Proteus 110 OD EC (a.i. deltamethrin+thiacloprid 10+100 g l^{-1}). The results show that all insecticides used in the trials were effective against *M. aeneus* and significantly reduced the amount of pollen beetle in sprayed plots, compared with unsprayed control plots 1-10 DAA. The differences between various insecticides and between their dose rates were not significant.

The efficacy of three dose rates of the new insecticide Steward (a.i. indoxacarb 300 g l^{-1}) from the chemical class of oxidiazines were tested in spring and winter oilseed rape crops in the 2008-2009 cropping seasons (Tables 5 and 6). The results show that all three doses of Steward were effective against pollen beetle in both spring and winter oilseed rape crops and significantly reduced the number of insects 1-7 DAA compared with the untreated plots. The efficacy of pyrethroid insecticide tau-fluvalinate (Mavrik) was on the same level as Steward.

Parameters		Untreated	Proteus 110 OD		Decis 50 EW		Fastac EC
			0.6 l ha^{-1}	0.75 l ha^{-1}	0.125 l ha^{-1}	0.15 l ha^{-1}	0.15 l ha^{-1}
		Meligethes aeneus					
		2005					
Adults per plant	before I spray	1.47 (10 June, GS 50)					
	1 DAA	1.24	0.00**	0.00**	0.00**	0.00**	0.00**
	4 DAA	1.47	0.00**	0.02**	0.02**	0.01**	0.05**
	7 DAA	1.13	0.18**	0.09**	0.15**	0.12**	0.26**
	10 DAA	1.95	0.39**	0.36**	0.35**	0.39**	0.30**
Biological efficacy %	1 DAA	-	100.0	100.0	100.0	100.0	100.0
	4 DAA	-	100.0	98.6	98.6	99.3	96.6
	7 DAA	-	84.1	92.0	86.7	89.4	77.0
	10 DAA	-	80.0	81.5	82.0	80.0	84.6
Adults per plant	before II spray	2.23 (22 June, GS 57)					
	1 DAA	3.00	0.17**	0.11**	0.06**	0.04**	0.40**
	4 DAA	1.11	0.14**	0.11**	0.19**	0.08**	0.31**
	7 DAA	1.03	0.17**	0.17**	0.14**	0.08**	0.18**
Biological efficacy %	1 DAA	-	94.3	96.3	98.0	98.7	86.7
	4 DAA	-	87.4	90.1	82.9	92.8	72.1
	7 DAA	-	83.5	83.5	86.4	92.2	82.5
		2006					
Adults per plant	before spray	1.10 (21 June, GS 53)					
	1 DAA	0.92	0.77	0.01**	0.03**	0.02**	0.03**
	4 DAA	1.11	0.05**	0.18**	0.03**	0.03**	0.07**
	7 DAA	1.29	0.19**	0.19**	0.22**	0.19**	0.19**
	10 DAA	1.24	0.17**	0.18**	0.12**	0.18**	0.14**
Biological efficacy %	1 DAA	-	16.3	98.9	96.7	97.8	96.7
	4 DAA	-	95.5	83.8	97.3	97.3	93.7
	7 DAA	-	85.3	85.3	83.0	85.3	85.3
	10 DAA	-	86.3	85.5	90.3	85.5	88.7

DAA – days after application; asterisks (*,**) denote significant difference at P≤0.05, P≤0.01 probability levels, respectively, using Fisher's least significant difference test; data were log(X+1) transformed before analysis, but non-transformed data are presented

Table 2. The effect of insecticides Proteus 110 OD (a.i. deltamethrin+thiacloprid 10+100 g l^{-1}), Decis 50 EW (a.i. deltamethrin 50 g l^{-1}) and Fastac EC (a.i. alpha-cypermethrin 100 g l^{-1}) on the number of pollen beetle (*Meligethes aeneus*) adults per plant in spring oilseed rape in 2005 and 2006.

Parameters		Untreated	Pyrinex Supreme			Bulldock 025 EC		Proteus 110 OD
			0.75 l ha^{-1}	1.0 l ha^{-1}	1.25 l ha^{-1}	0.225 l ha^{-1}	0.3 l ha^{-1}	0.75 l ha^{-1}
		Meligethes aeneus						
		2007						
Adults per plant	before I spray	0.77 (1 June, GS 50)						
	1 DAA	0.10	0.00**	0.00**	0.00**	0.00**	0.00**	0.00**
	4 DAA	1.13	0.22**	0.22**	0.20**	0.30**	0.24**	0.21**
Biological efficacy %	1 DAA	-	100.0	100.0	100.0	100.0	100.0	100.0
	4 DAA	-	80.5	80.5	82.3	73.4	78.8	81.4
Adults per plant	before II spray	2.57 (7 June, GS 55-57)						
	1 DAA	2.22	0.71**	0.65**	0.39**	0.52**	0.78**	0.53**
	4 DAA	1.37	0.05**	0.00**	0.00**	0.10**	0.08**	0.02**
	7 DAA	0.92	0.03**	0.00**	0.00**	0.02**	0.02**	0.01**
Biological efficacy %	1 DAA	-	68.0	70.7	82.4	76.6	64.9	76.1
	4 DAA	-	96.4	100.0	100.0	92.7	94.2	98.5
	7 DAA	-	96.7	100.0	100.0	97.8	97.8	98.9
		2008						
Adults per plant	before spray	1.37 (5 June, GS 39)						
	1 DAA	0.31	0.00**	0.00**	0.00**	0.00**	0.00**	0.00**
	4 DAA	0.40	0.00**	0.00**	0.00**	0.00**	0.00**	0.00**
	7 DAA	0.67	0.00**	0.00**	0.00**	0.00**	0.03**	0.24**
	10 DAA	0.73	0.34**	0.17**	0.19**	0.16**	0.23**	0.23**
	14 DAA	0.77	0.29**	0.18**	0.24**	0.30**	0.21**	0.23**
Biological efficacy %	1 DAA	-	100.0	100.0	100.0	100.0	100.0	100.0
	4 DAA	-	100.0	100.0	100.0	100.0	100.0	100.0
	7 DAA	-	100.0	100.0	100.0	100.0	95.5	64.2
	10 DAA	-	53.4	76.7	74.0	78.1	68.5	68.5
	14 DAA	-	62.3	76.6	68.8	61.0	72.7	70.1

DAA – days after application; asterisks (*,**) denote significant difference at P≤0.05, P≤0.01 probability levels, respectively, using Fisher's least significant difference test; data were log(X+1) transformed before analysis, but non-transformed data are presented

Table 3. The effect of insecticides Pyrinex Supreme (a.i. beta-cyfluthrin+chlorpyrifos 12+250 g l^{-1}), Bulldock (a.i. beta-cyfluthrin 25 g l^{-1}) and Proteus 110 OD EC (a.i. deltamethrin+thiacloprid 10+100 g l^{-1}) on the number of pollen beetle (*Meligethes aeneus*) adults per plant in spring oilseed rape in 2007 and 2008.

Parameters		Untreated	Pyrinex Supreme			Bulldock 025 EC		Proteus 110 OD
			0.75 l ha^{-1}	1.0 l ha^{-1}	1.25 l ha^{-1}	0.225 l ha^{-1}	0.3 l ha^{-1}	0.75 l ha^{-1}
		Meligethes aeneus						
		2007						
Adults per plant	before spray	1.80 (24 April, GS 55)						
	1 DAA	2.20	0.03**	0.04**	0.02**	0.02**	0.02**	0.04**
	4 DAA	1.84	0.19**	0.14**	0.04**	0.12**	0.05**	0.08**
	7 DAA	0.66	0.14**	0.01**	0.00**	0.04**	0.02**	0.01**
	10 DAA	1.61	1.09**	0.93**	0.74**	0.59**	0.81**	0.79**
Biological efficacy %	1 DAA	-	98.6	98.2	99.1	99.1	99.1	98.2
	4 DAA	-	89.7	92.4	97.8	93.5	97.3	95.6
	7 DAA	-	78.8	98.5	100.0	93.9	97.0	98.5
	10 DAA	-	32.3	42.2	54.0	63.4	49.7	50.9
		2008						
Adults per plant	before I spray	2.70 (15 April, GS 50-51)						
	1 DAA	0.28	0.00**	0.00**	0.00**	0.00**	0.00**	0.00**
	4 DAA	0.22	0.00**	0.00**	0.00**	0.00**	0.00**	0.00**
	7 DAA	0.49	0.03**	0.03**	0.01**	0.02**	0.05**	0.06**
	10 DAA	0.48	0.06**	0.05**	0.06**	0.06**	0.03**	0.08**
Biological efficacy %	1 DAA	-	100.0	100.0	100.0	100.0	100.0	100.0
	4 DAA	-	100.0	100.0	100.0	100.0	100.0	100.0
	7 DAA	-	93.9	93.9	98.0	95.9	89.8	87.8
	10 DAA	-	87.5	89.6	87.5	87.5	93.8	83.3
Adults per plant	before II spray	1.30 (28 April, GS 53-55)						
	1 DAA	0.29	0.00**	0.01**	0.02**	0.01**	0.00**	0.00**
	4 DAA	0.59	0.03**	0.03**	0.04**	0.08**	0.03**	0.04**
	7 DAA	0.17	0.01**	0.08**	0.01**	0.03**	0.03**	0.00**
	10 DAA	0.28	0.03**	0.04**	0.02**	0.05**	0.04**	0.04**
Biological efficacy %	1 DAA	-	100.0	96.6	93.1	96.6	100.0	100.0
	4 DAA	-	94.9	94.9	93.2	86.4	94.9	93.2
	7 DAA	-	94.1	52.9	94.1	82.4	82.4	100.0
	10 DAA	-	89.3	85.7	92.9	82.1	85.7	85.7

DAA – days after application; asterisks (*,**) denote significant difference at P≤0.05, P≤0.01 probability levels, respectively, using Fisher's least significant difference test; data were log(X+1) transformed before analysis, but non-transformed data are presented

Table 4. The effect of insecticides Pyrinex Supreme (a.i. beta-cyfluthrin+chlorpyrifos 12+250 g l^{-1}), Bulldock (a.i. beta-cyfluthrin 25 g l^{-1}) and Proteus 110 OD (a.i. deltamethrin+thiacloprid 10+100 g l^{-1}) on the number of pollen beetle (*Meligethes aeneus*) adults per plant in winter oilseed rape in 2007 and 2008.

Parameters		Untreated	Steward EC			Mavrik 2 F
			0.0425 kg ha^{-1}	0.0625 kg ha^{-1}	0.085 kg ha^{-1}	0.3 l ha^{-1}
		Meligethes aeneus				
		2008				
Adults per plant before	before spray	1.36 (5 June, GS 39)				
	1 DAA	0.36	0.01**	0.02**	0.02**	0.01**
	4 DAA	0.43	0.00**	0.00**	0.00**	0.00**
	7 DAA	0.54	0.00**	0.21**	0.04**	0.07**
	10 DAA	0.25	0.25	0.21	0.25	0.21
	14 DAA	0.89	0.23**	0.21**	0.29**	0.32**
Biological efficacy %	1 DAA	-	97.2	94.4	94.4	97.2
	4 DAA	-	100.0	100.0	100.0	100.0
	7 DAA	-	100.0	61.1	92.6	87.0
	10 DAA	-	0.0	16.0	0.0	16.0
	14 DAA	-	74.2	76.4	67.4	64.0
		2009				
Adults per plant before	before spray	2.50 (13 June, GS 53)				
	1 DAA	2.90	0.24**	0.46**	0.57**	0.00**
	4 DAA	2.29	0.12**	0.15**	0.19**	0.00**
	7 DAA	1.30	0.12**	0.06**	0.11**	0.06**
	10 DAA	0.66	0.15**	0.12**	0.15**	0.15**
Biological efficacy %	1 DAA	-	91.7	84.1	80.3	100.0
	4 DAA	-	94.8	93.4	91.7	100.0
	7 DAA	-	90.8	95.4	91.5	95.4
	10 DAA	-	77.3	81.8	77.3	77.3
DAA – days after application; asterisks (*,**) denote significant difference at P≤0.05, P≤0.01 probability levels, respectively, using Fisher's least significant difference test; data were log(X+1) transformed before analysis, but non-transformed data are presented						

Table 5. The effect of insecticides Steward EC (a.i. indoxacarb 300 g l^{-1}) and Mavrik 2 F (a.i. tau-fluvalinate 240 g l^{-1}) on the number of pollen beetle (*Meligethes aeneus*) adults per plant in spring oilseed rape in 2008 and 2009.

Parameters		Untreated	Steward EC			Mavrik 2 F
			0.0425 kg ha^{-1}	0.0625 kg ha^{-1}	0.085 kg ha^{-1}	0.3 l ha^{-1}
		Meligethes aeneus				
		2008				
Adults per plant	before I spray	1.16 (25 April, GS 52-53)				
	1 DAA	0.08	0.01**	0.01**	0.02**	0.04
	4 DAA	0.45	0.17**	0.08**	0.24**	0.09**
	7 DAA	1.00	0.52**	0.45**	0.50**	0.21**
Biological efficacy %	1 DAA	-	87.5	87.5	75.0	50.0
	4 DAA	-	62.2	82.2	46.7	80.0
	7 DAA	-	48.0	55.0	50.0	79.0
Adults per plant	before II spray	0.54 (2 May, GS 57-59)				
	1 DAA	0.19	0.03**	0.01**	0.00**	0.01**
	4 DAA	0.38	0.00**	0.01**	0.03**	0.00**
Biological efficacy %	1 DAA	-	84.2	94.7	100.0	94.7
	4 DAA	-	100.0	97.4	92.1	100.0
		2009				
Adults per plant	before spray	2.50 (4 May, GS 57)				
	1 DAA	0.74	0.27**	0.24**	0.31**	0.00**
	4 DAA	0.81	0.31**	0.25**	0.25**	0.20**
	7 DAA	0.37	0.14**	0.14**	0.15**	0.09**
	10 DAA	0.21	0.14	0.15	0.11*	0.09*
Biological efficacy %	1 DAA	-	63.5	67.6	58.1	100.0
	4 DAA	-	61.7	69.1	69.1	75.3
	7 DAA	-	62.2	62.2	59.5	75.7
	10 DAA	-	33.3	28.6	47.6	57.1

DAA – days after application; asterisks (*,**) denote significant difference at P≤0.05, P≤0.01 probability levels, respectively, using Fisher's least significant difference test; data were log(X+1) transformed before analysis, but non-transformed data are presented

Table 6. The effect of insecticides Steward EC (a.i. indoxacarb 300 g l^{-1}) and Mavrik 2 F (a.i. tau-fluvalinate 240 g l^{-1}) on the number of pollen beetle (*Meligethes aeneus*) adults per plant in winter oilseed rape in 2008 and 2009.

3.2 The effect of insecticides on the pod infestation by larvae of *Ceutorhynchus obstrictus* and *Dasineura brassicae* and on the number of stems damaged by *C. pallidactylus* larvae in spring and winter oilseed rape crops

Little is known about the harmfullness of stem and pod pests in winter and spring rape, earlier it was published that *Ceutorhynchus* species were of minor importance in rape crops in Lithuania (Tamutis, 1997). However, it seems that increasing production area of spring and winter oilseed rape has increased the incidence and severity of stem and pod pests in both crops. Our research evidenced that damage caused by larvae of *C. pallidactylus* was

higher in winter rape, compared with spring rape. It was estimated, that during experimental period, *C. pallidactylus* larvae damaged 18.3 - 87.0% of winter rape and up to 32.5% of spring rape stems (Tables 7 – 11).

Parameters	Untreated	Proteus 110 OD		Decis 50 EW		Fastac EC
		0.6 l ha^{-1}	0.75 l ha^{-1}	0.125 l ha^{-1}	0.15 l ha^{-1}	0.15 l ha^{-1}
Ceutorhynchus obstrictus						
2005						
Infested pods %	0.3	0.4	0.0	0.1	0.1	0.4
Reduction %	-	0.0	100.0	66.7	66.7	0.0
Larvae per pod	0.01	0.01	0.00	0.01	0.01	0.01
Reduction %	-	0.0	100.0	0.0	0.0	0.0
2006						
Infested pods %	6.3	5.3*	5.0**	4.9**	4.8**	5.3*
Reduction %	-	15.9	20.6	22.2	23.8	15.9
Larvae per pod	0.06	0.04**	0.03**	0.03**	0.03**	0.04**
Reduction %	-	33.3	50.0	50.0	50.0	33.3
Dasineura brassicae						
2005						
Infested pods %	8.3	5.4**	4.1**	5.4**	4.8**	5.8**
Reduction %	-	34.9	50.6	34.9	42.2	30.1
Larvae per pod	2.31	0.70**	0.41**	0.75**	0.75**	0.79**
Reduction %	-	69.7	82.2	67.5	67.5	65.8
2006						
Infested pods %	3.3	3.0	3.1	3.1	3.0	3.1
Reduction %	-	9.1	6.1	6.1	9.1	6.1
Larvae per pod	0.61	0.37	0.42	0.39	0.35	0.38
Reduction %	-	39.3	31.2	36.1	42.6	37.7
Ceutorhyncus pallidactylus						
2005						
Damaged stems %	32.5	19.0**	17.3**	19.2**	18.4**	18.8**
Reduction %	-	41.5	46.8	40.9	43.4	42.2
Larvae exit holes per stem	0.33	0.19**	0.17**	0.19**	0.18**	0.19**
Reduction %	-	42.4	48.5	42.4	45.4	42.4
2006						
Damaged stems %	16.3	10.4**	8.7**	9.6**	8.3**	11.7**
Reduction %	-	36.2	46.6	41.1	49.1	28.2
Larvae exit holes per stem	0.17	0.11*	0.09*	0.01*	0.08*	0.12*
Reduction %	-	35.3	47.1	94.1	52.9	29.4
Asterisks (*,**) denote a significant difference at P≤0.05, P≤0.01 probability levels, respectively, using Fisher's least significant difference test; data were arcsin $\sqrt{X\%}$ transformed before analysis, but non-transformed data are presented						

Table 7. The effect of insecticides Proteus 110 OD (a.i. deltamethrin+thiacloprid 10+100 g l^{-1}), Decis 50 EW (a.i. deltamethrin 50 g l^{-1}) and Fastac EC (a.i. alpha-cypermethrin 100 g l^{-1}) on the pod infestation by larvae of cabbage seedpod weevil (*Ceutorhynchus obstrictus*) and brassica pod midge (*Dasineura brassicae*) and on the number of stems damaged by cabbage stem weevil (*Ceutorhyncus pallidactylus*) larvae in spring oilseed rape in 2005 and 2006.

Parameters	Untreated	Pyrinex Supreme			Bulldock 025 EC		Proteus 110 OD
		0.75 l ha^{-1}	1.0 l ha^{-1}	1.25 l ha^{-1}	0.225 l ha^{-1}	0.3 l ha^{-1}	0.75 l ha^{-1}
Ceutorhynchus obstrictus							
2007							
Infested pods %	0.3	0.3	0.1	0.0	0.0	0.6	0.8
Reduction %	-	0.0	66.7	100.0	100.0	0.0	0.0
Larvae per pod	<0.01	<0.01	<0.01	0.00	0.00	0.01	0.01
Reduction %	-	0.0	0.0	0.0	0.0	0.0	0.0
2008							
Infested pods %	2.0	0.3**	0.3**	1.0	1.5	1.3	0.5**
Reduction %	-	85.0	85.0	50.0	25.0	35.0	75.0
Larvae per pod	0.66	0.12	0.02**	0.36	0.84	0.62	0.17
Reduction %	-	81.8	97.0	45.4	0.0	6.1	74.2
Dasineura brassicae							
2007							
Infested pods %	3.6	4.4	2.8	2.8	3.5	4.1	2.1
Reduction %	-	0.0	22.2	22.2	2.8	0.0	41.7
Larvae per pod	1.30	1.38	0.90	0.78	1.24	1.44	0.74
Reduction %	-	0.0	30.8	40.0	4.6	0.0	43.1
2008							
Infested pods %	3.5	1.5	1.0*	0.0**	1.8	1.8	1.0*
Reduction %	-	57.1	71.4	100.0	48.6	48.6	71.4
Larvae per pod	0.04	0.02	0.01**	0.00**	0.02	0.02	0.01**
Reduction %	-	50.0	75.0	100.0	50.0	50.0	75.0
Ceutorhyncus pallidactylus							
2007							
Damaged stems %	11.3	12.5	11.9	11.7	9.6	8.8	11.7
Reduction %	-	0.0	0.0	0.0	15.0	22.1	0.0
Larvae exit holes per stem	0.11	0.13	0.12	0.12	0.08	0.11	0.12
Reduction %	-	0.0	0.0	0.0	27.3	0.0	0.0
2008							
Damaged stems %	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Reduction %	-	0.0	0.0	0.0	0.0	0.0	0.0
Larvae exit holes per stem	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Reduction %	-	0.0	0.0	0.0	0.0	0.0	0.0

Asterisks (*,**) denote a significant difference at P≤0.05, P≤0.01 probability levels, respectively, using Fisher's least significant difference test; data were arcsin $\sqrt{X\%}$ transformed before analysis, but non-transformed data are presented

Table 8. The effect of insecticides Pyrinex Supreme (a.i. beta-cyfluthrin+chlorpyrifos 12+250 g l^{-1}), Bulldock 025 EC (a.i. beta-cyfluthrin 25 g l^{-1}) and Proteus 110 OD (a.i. deltamethrin+thiacloprid 10+100 g l^{-1}) on the pod infestation by larvae of cabbage seedpod weevil (*Ceutorhynchus obstrictus*) and brassica pod midge (*Dasineura brassicae*) and on the number of stems damaged by cabbage stem weevil (*Ceutorhyncus pallidactylus*) larvae in spring oilseed rape in 2007 and 2008.

Parameters	Untreated	Pyrinex Supreme			Bulldock 025 EC		Proteus 110 OD
		0.75 l ha^{-1}	1.0 l ha^{-1}	1.25 l ha^{-1}	0.225 l ha^{-1}	0.3 l ha^{-1}	0.75 l ha^{-1}
Ceutorhynchus obstrictus							
2007							
Infested pods %	1.0	1.5	0.3	0.6	0.8	0.3	0.00*
Reduction %	-	0.0	70.0	40.0	20.0	70.0	100.0
Larvae per pod	0.01	0.02	0.00	0.01	0.01	0.02	0.00
Reduction %	-	0.0	100.0	0.0	0.0	0.0	100.0
2008							
Infested pods %	5.5	1.8*	1.3*	2.5*	1.5*	1.3*	1.8*
Reduction %	-	67.3	76.4	54.5	72.7	76.4	67.3
Larvae per pod	0.06	0.02*	0.01*	0.03*	0.02*	0.01*	0.02*
Reduction %	-	66.7	83.3	50.0	66.7	83.3	66.7
Dasineura brassicae							
2007							
Infested pods %	13.4	9.9*	7.1**	9.5*	11.5	9.3*	8.0**
Reduction %	-	26.1	47.0	29.1	14.2	30.6	40.3
Larvae per pod	1.48	0.90*	0.64**	0.61**	1.20	0.96	0.72**
Reduction %	-	39.2	56.8	58.8	18.9	35.1	51.4
2008							
Infested pods %	8.5	5.5*	3.0**	3.0**	3.0**	4.8**	4.8**
Reduction %	-	35.3	64.7	64.7	64.7	43.5	43.5
Larvae per pod	0.83	0.21**	0.15**	0.11**	0.10**	0.17**	0.23**
Reduction %	-	74.70	81.9	86.8	88.0	79.5	72.3
Ceutorhyncus pallidactylus							
2007							
Damaged stems %	18.3	7.1**	4.6**	6.3**	7.1**	7.1**	7.5**
Reduction %	-	61.2	74.9	65.6	61.2	61.2	59.0
Larvae exit holes per stem	0.23	0.09**	0.05**	0.07**	0.09**	0.09**	0.08**
Reduction %	-	60.9	78.3	69.6	60.9	60.9	65.2
2008							
Damaged stems %	20.0	6.3**	1.3**	1.2**	0.0**	5.0**	0.0**
Reduction %	-	68.5	93.5	94.0	100.0	75.0	100.0
Larvae exit holes per stem	0.21	0.06**	0.02**	0.01**	0.00**	0.05**	0.00**
Reduction %	-	71.4	90.5	95.2	100.0	76.2	100.0

Asterisks (*,**) denote a significant difference at P≤0.05, P≤0.01 probability levels, respectively, using Fisher's least significant difference test; data were arcsin $\sqrt{X\%}$ transformed before analysis, but non-transformed data are presented

Table 9. The effect of insecticides Pyrinex Supreme (a.i. beta-cyfluthrin+chlorpyrifos 12+250 g l^{-1}), Bulldock (a.i. beta-cyfluthrin 25 g l^{-1}) and Proteus 110 OD (a.i. deltamethrin+thiacloprid 10+100 g l^{-1}) on the pod infestation by larvae of cabbage seedpod weevil (*Ceutorhynchus obstrictus*) and brassica pod midge (*Dasineura brassicae*) and on the number of stems damaged by cabbage stem weevil (*Ceutorhyncus pallidactylus*) larvae in winter oilseed rape in 2007 and 2008.

In the 2008 cropping season, in untreated plots of winter oilseed rape field we found 20.0 – 35.0% of stems damaged by *C. pallidactylus* larvae (Tables 9 and 11) while in the field of spring oilseed rape such stems were not observed at all (Tables 8 and 10). The highest amount of damaged stems (87.0%) was estimated in winter rape during the 2009 cropping season.

Pod pests – *C. obstrictus* and *Dasineura brassicae* were estimated to be of economic importance both in winter and spring oilseed rape. At the pod development stage (GS 73) *C. obstrictus* larvae were found in 6.3% of spring rape pods in untreated plots in the 2006 and 2009 cropping seasons (Tables 7 and 10) and the highest percentage of winter rape pods infested by this insect (5.5%) was observed in 2008 (Table 9). The larvae of *D. brassicae* were detected in the assessed pods with a higher frequency, compared to the *C. obstrictus*. Therefore, during experimental period, there were found 7.5 - 13.4% of winter rape and 1.8 - 8.3% of spring rape pods with the presence of *D. brassicae* larvae inside the pods. The average number of larvae/pod in winter rape in untreated plots ranged from 0.54 to 1.5 and in spring rape from 0.2 to 2.3 larvae/assessed pod. The number of *C. obstrictus* larvae/pod in untreated control plots was much lower, compared to the abundance of *D. brassicae* larvae. In general, this is not very high infestation of pods, compared to the data obtained in Czech Republic, where up to 86.0% of pods were found infested with larvae of *D. brassicae* in the control plots (Pavela et al., 2007).

All tested insecticides, used for the control of *M. aeneus*, also decreased the number of pods infested by larvae of *C. obstrictus* and *D. brassicae* and the number of stems, damaged by *C. pallidactylus*, in some cases these decreases were essential. Insecticide application in spring oilseed rape significantly decreased the number of stems damaged by *C. pallidactylus* larvae, the reduction of damaged stems was 40.9 - 46.8% during 2005 (two insecticide applications, at GS 50 and GS 57) and 36.2 – 49.1% during the 2006 cropping season (insecticide application at GS 53) (Table 7). However, the efficacy of insecticides on the control of *C. obstrictus* and *D. brassicae* varied during this experimental period. Insecticides essentially prevented pod infestation by *C. obstrictus* larvae and reduced the number of larvae/pod in 2006 and by *D. brassicae* larvae in 2005. Insecticides were not effective, when the infestation of pods was low in the control plots (in 2005 only 0.3% of *C. obstrictus* larvae-infested and in 2006 3.3% of *D. brassicae* larvae-infested pods). Obviously, the application time of insecticides was focused on the spray threshold of pollen beetle, while the best treatment time for the control of brassica pod midge is recorded to be later – at the flowering growth stage (GS 65-67) (Pavela et al., 2007). Our results show that the efficacy of Proteus (neonicotinoid class) was on the same level as pyrethroids (Decis and Fastac) in 2005 and 2006. However, other researchers have reported that the application of neonicotinoid insecticides had exhibited the highest efficacy in the control of the brassica pod midge (Pavela et al., 2007).

The infestation of winter rape pods with larvae of *C. obstrictus* and *D. brassicae* in the control untreated plots during the 2007 and 2008 cropping seasons was much higher, compared with spring oilseed rape pods (Table 8). In spring oilseed rape, Pyrinex Supreme (a.i. beta-cyfluthrin+chlorpyrifos 12+250 g l^{-1}), Bulldock 025 EC (a.i. beta-cyfluthrin 25 g l^{-1}) and Proteus 110 OD (a.i. deltamethrin+thiacloprid 10+100 g l^{-1}) used twice in 2007 against pollen beetle (at GS 50 and GS 55-57) had no significant effect on the pod infestation with *C. obstrictus* and *D. brassicae* larvae. However, a single early application of insecticides in 2008 (GS 39) effectively prevented pod infestation. Insecticides had no effect on the number of damaged stems by *C. pallidactylus* larvae in spring oilseed rape in both cropping seasons.

Parameters	Untreated	Steward EC			Mavrik 2 F
		0.0425 kg ha^{-1}	0.0625 kg ha^{-1}	0.085 kg ha^{-1}	0.3 l ha^{-1}
Ceutorhynchus obstrictus					
2008					
Infested pods %	1.8	0.8	1.3	1.5	2.0
Reduction %	-	55.6	27.8	16.7	0.0
Larvae per pod	0.02	0.01	0.01	0.02	0.07
Reduction %	-	50.0	50.0	0.0	0.0
2009					
Infested pods %	6.3	2.8	3.3	2.0**	4.5
Reduction %	-	55.6	47.6	68.2	28.6
Larvae per pod	0.06	0.03*	0.03*	0.03*	0.05
Reduction %	-	50.0	50.0	50.0	16.7
Dasineura brassicae					
2008					
Infested pods %	1.8	0.5	1.0	1.8	2.0
Reduction %	-	72.2	44.4	0.0	0.0
Larvae per pod	0.18	0.06	0.04	0.07	0.34
Reduction %	-	66.7	77.8	61.1	0.0
2009					
Infested pods %	8.5	2.5**	2.0**	2.0**	3.3**
Reduction %	-	70.6	76.5	76.5	61.2
Larvae per pod	1.19	0.24**	0.19**	0.21**	0.39**
Reduction %	-	79.8	84.0	82.4	67.2
Ceutorhyncus pallidactylus					
2008					
Damaged stems %	0.0	0.0	0.0	0.0	0.0
Reduction %	-	0.0	0.0	0.0	0.0
Larvae exit holes per stem	0.00	0.00	0.00	0.00	0.00
Reduction %	-	0.0	0.0	0.0	0.0
2009					
Damaged stems %	22.0	0.0**	7.0**	5.0**	1.0**
Reduction %	-	100.0	68.2	77.3	95.5
Larvae exit holes per stem	0.31	0.00**	0.07**	0.08**	0.02**
Reduction %	-	100.0	77.4	74.2	93.6
Asterisks (*,**) denote a significant difference at P≤0.05, P≤0.01 probability levels, respectively, using Fisher's least significant difference test; data were arcsin $\sqrt{X\%}$ transformed before analysis, but non-transformed data are presented					

Table 10. The effect of insecticides Steward EC (a.i. indoxacarb 300 g l^{-1}) and Mavrik 2 F (a.i. tau-fluvalinate 240 g l^{-1}) on the pod infestation with larvae of cabbage seedpod weevil (*Ceutorhynchus obstrictus*) and brassica pod midge (*Dasineura brassicae*) and on the number of stems damaged by cabbage stem weevil (*Ceutorhyncus pallidactylus*) larvae in spring oilseed rape during the 2008 and 2009 cropping seasons.

Parameters	Untreated	Steward EC			Mavrik 2 F
		0.0425 kg ha^{-1}	0.0625 kg ha^{-1}	0.085 kg ha^{-1}	0.3 l ha^{-1}
Ceutorhynchus obstrictus					
2008					
Infested pods %	4.3	0.8**	1.8	2.5	0.8**
Reduction %	-	81.4	58.1	41.9	81.4
Larvae per pod	0.13	0.01*	0.02	0.03	0.01*
Reduction %	-	92.3	84.6	76.9	92.3
2009					
Infested pods %	2.5	2.1	1.8	2.0	1.6
Reduction %	-	16.0	28.0	20.0	36.0
Larvae per pod	0.03	0.02	0.02	0.02	0.02
Reduction %	-	33.3	33.3	33.3	33.3
Dasineura brassicae					
2008					
Infested pods %	7.5	2.8**	3.3**	3.0**	2.5**
Reduction %	-	62.7	56.0	60.0	66.7
Larvae per pod	0.54	0.11**	0.13**	0.10**	0.08**
Reduction %	-	79.6	75.9	81.5	85.2
2009					
Infested pods %	7.8	2.0**	4.0*	2.1**	2.0**
Reduction %	-	74.4	48.7	73.1	74.4
Larvae per pod	0.70	0.11**	0.19**	0.08**	0.12**
Reduction %	-	84.3	72.9	88.6	82.9
Ceutorhyncus pallidactylus					
2008					
Damaged stems %	35.0	15.0**	20.0**	25.0	26.3
Reduction %	-	57.1	42.9	28.6	24.9
Larvae exit holes per stem	0.54	0.19**	0.24**	0.29**	0.33*
Reduction %	-	64.8	55.6	46.3	38.9
2009					
Damaged stems %	87.0	76.0*	67.0**	67.0**	71.0**
Reduction %	-	12.6	23.0	23.0	18.4
Larvae exit holes per stem	2.05	1.19**	1.03**	.97**	1.04**
Reduction %	-	42.0	49.8	52.7	49.3

Asterisks (*,**) denote significant difference at P≤0.05, P≤0.01 probability levels, respectively, using Fisher's least significant difference test; data were arcsin $\sqrt{X\%}$ transformed before analysis, but non-transformed data are presented

Table 11. The effect of insecticides Steward EC (a.i. indoxacarb 300 g l^{-1}) and Mavrik 2 F (a.i. tau-fluvalinate 240 g l^{-1}) on the pod infestation by larvae of cabbage seedpod weevil (*Ceutorhynchus obstrictus*) and brassica pod midge (*Dasineura brassicae*) and on the number of stems damaged by cabbage stem weevil (*Ceutorhyncus pallidactylus*) larvae in winter oilseed rape during the 2008 and 2009 cropping seasons.

As it has been mentioned above, winter oilseed rape crop was more heavily infested with pod and stem pest larvae, compared with spring rape. The insecticides for *M. aeneus* control in winter rape in 2007 were used once (GS 55) and in 2008 twice (GS 50-51 and GS 53-55). All insecticides (Pyrinex Supreme (a.i. beta-cyfluthrin+chlorpyrifos 12+250 g l^{-1}), Bulldock 025 EC (a.i. beta-cyfluthrin 25 g l^{-1}) and Proteus 110 OD (a.i. deltamethrin+thiacloprid 10+100 g l^{-1}) used at all dose rates effectively reduced pod infestation with larvae of *C. obstrictus* and *D. brassicae*, also the number of stems damaged by *C. pallidactylus* larvae (Table 9). Due to different insecticide application the number of pods infested with *C. obstrictus* larvae was reduced by 0 - 100.0% and by 54.5 - 76.4% and pod infestation with *D. brassicae* larvae was reduced by 14.2 – 47.0% and by 35.3 – 64.7 % in the 2007 and 2008 cropping seasons, respectively.

The number of stems damaged by *C. pallidactylus* larvae was reduced by 59.0 – 74.9% in 2007 and by 68.5 – 100.0% in 2008. It seems that the application of insecticides against pollen beetle twice in 2008 gave a more effective control of also pod and stem pests, compared with a single application in 2007. However, significant differences between the different class insecticides were not revealed.

Insecticides Steward EC (a.i. indoxacarb 300 g l^{-1}) and Mavrik 2 F (a.i. tau-fluvalinate 240 g l^{-1}) were used for the control of pollen beetle and other pests in spring and winter oilseed rape in the 2008 and 2009 cropping seasons. The infestation of pods with larvae of *C. obstrictus* and *D. brassicae* in the control untreated plots in winter oilseed rape during the 2008 cropping season was higher, compared with spring rape; however, in 2009 higher infestation in spring rape was observed (Tables 10, 11). In spring rape, insecticides were used once in the 2008 and 2009 cropping seasons, at GS 39 and GS 53, respectively. The results show higher infestation of pods with *C. obstrictus* and *D. brassicae* larvae and stems with *C. pallidactylus* larvae in 2009, compared with the 2008 cropping season. Due to the application of insecticide Steward, pod infestation with *C. obstrictus* larvae was reduced by 16.7-55.6% and by 47.6 – 68.2% in 2008 and 2009, respectively; however, this reduction was not essential (Table 10). Pyrethroid insecticide Mavrik was less effective in preventing pod infestation with *C. obstrictus* larvae, compared with Steward (indoxacarb chemical class). The effect of different dose rates of Steward on *D. brassicae* was very variable in 2008, although in 2009 all three dose rates of Steward showed high efficacy in the reduction of pods infested with larvae of *D. brassicae.* Insecticides Steward and Mavrik also effectively reduced the number of stems, damaged by *C. pallidactylus* larvae in the 2009 cropping season (reduction was 68.2 – 100.0%).

In winter rape, insecticides were used twice in 2008 (at GS 52-53 and GS 57-59) and once in the 2009 cropping season, at GS 57. The results show that pod infestation with *C. obstrictus* larvae in untreated control plots was much higher in 2008, compared with 2009 (4.3 and 2.5%, respectively) and pod infestation with larvae of *D. brassicae* was very similar in both experimental years (7.5 and 7.8%). Very high number of stems damaged by *C. pallidactylus* larvae (87.0%) was observed in 2009, while only 35.0% of such stems were found in the 2008 cropping season. Insecticide Steward reduced pod infestation with *C. obstrictus* larvae by 41.9 – 81.4% and by 16.0 – 28.0% in 2008 and 2009, respectively; however, this reduction in most cases was not essential (Table 11). Pod infestation with *D. brassicae* larvae was controlled more effectively. The reduction of infested pods and the number of larvae/pod was essential in both cropping seasons. The efficacy of Steward and of Mavrik was on the same level and no remarkable differences were obtained between the efficacy of insecticides from different chemical classes. Other researchers have reported that the pyrethroid

insecticides were more effective than endosulfan and methyl parathion at reducing adult numbers and preventing pod infestation by larvae (Buntin, 1999a). Different dose rates of Steward showed very similar efficacy in controlling pod infestation with *C. obstrictus* and *D. brassicae* larvae. Usually optimal time for controlling of pod pests is during flowering (Buntin, 1999a); however our research evidenced that insecticide application against pollen beetle during inflorescence emergence growth stage also effectively prevented pod infestation with larvae of *C. obstrictus* and *D. brassicae*.

Insecticide application at inflorescence emergence growth stage (GS 52-59) also reduced the number of stems damaged by *C. pallidactylus* larvae. The highest reduction (42.9 – 57.1%) was obtained in the 2008 cropping season where the lower dose rates of Steward showed higher efficacy and this reduction was essential, compared with the untreated plots. In 2009, when winter rape stem infestation with this pest was very high, the number of infested stems in insecticide treated plots was reduced only by 12.6 – 23.0%, however this reduction was essential in all cases. Despite the fact that insecticides were effective against *C. pallidactylus*, the number of damaged stems remained very high (67.0 -76.0%) in the insecticide treated plots in winter rape during the 2009 cropping season. It seems that the application of insecticides at GS 52-59 was not optimal for the effective control of *C. pallidactylus*.

3.3 The effect of insecticides on the productivity of spring and winter oilseed rape

Insecticide treatment had a positive effect on the seed yield of spring and winter oilseed rape; however, only in some cases substantial seed yield increase resulting from insecticide application was obtained. It seems that the effect of different insecticides on the productivity of spring and winter oilseed rape was highly dependent on the year. Insecticide Proteus (a.i. deltamethrin+thiacloprid) at a dose rate of 0.75 l ha^{-1} significantly ($P \leq 0.01$) increased the seed yield of spring rape in 2005 (by 19.7%), 2006 (25.7%) and in 2007 (21.6%) (Tables 12 and 13). Proteus application also provided a substantial increase in the seed yield of winter oilseed rape in the 2007 cropping season (the yield increased by 30.5%, compared with the unsprayed control) (Table 13). The higher dose rate of Proteus provided the higher seed yield increase. However, during the 2008 cropping season the seed yield obtained in Proteus sprayed plots was of the same level as in unsprayed control plots in both spring and winter oilseed rape.

Insecticide Pyrinex Supreme (a.i. beta-cyfluthrin+chlorpyrifos) belonging to the organophosphorus chemical class, used at three dose rates (0.75; 1.0 and 1.25 l ha^{-1}) provided substantial yield and TSW increase in spring and winter oilseed rape, but only in one of the two years. Application of this insecticide caused a 16.9 - 20.6% seed yield increase in the 2007 cropping season in spring rape and in winter rape 24.4 – 26.2%, compared with the untreated control plots (Table 13). Pyrethroids Decis (a.i. deltamethrin) and Fastac (a.i. alpha-cypermethrin) were used in the trials in the 2005 and 2006 cropping seasons and their application in all cases caused a substantial seed yield increase in spring oilseed rape (Table 12). The efficacy of Decis and Fastac was of the same level as Proteus, belonging to the neonicotinoid chemical class.

In the other group of trials, during the 2007 and 2008 cropping seasons another pyrethroid Bulldock (a.i. beta-cyfluthrin) was compared with the insecticides Pyrinex Supreme (organophosphorus chemical class) and Proteus (neonicotinoid chemical class). The results show that in both cropping seasons the effect of Bulldock on the seed yield of spring and winter oilseed rape was on the same level as in the plots, treated by Pyrinex Supreme and Proteus (Table 13).

Parameters	Untreated	Proteus 110 OD		Decis 50 EW		Fastac EC
		0.6 l ha^{-1}	0.75 l ha^{-1}	0.125 l ha^{-1}	0.15 l ha^{-1}	0.15 l ha^{-1}
2005						
Seed yield, kg ha^{-1}	2254	2532	2698**	2769**	2682**	2519
% to untreated	100.0	112.4	119.7	122.9	119.0	111.8
TSW, g	3.67	3.77	3.76	3.72	3.73	3.73
% to untreated	100.0	102.7	102.4	101.2	101.4	101.4
2006						
Seed yield, kg ha^{-1}	1392	1742**	1749**	1680**	1735**	1618**
% to untreated	100.0	125.1	125.7	120.7	124.7	116.3
TSW, g	4.50	4.63**	4.63**	4.65**	4.65**	4.55
% to untreated	100.0	103.0	103.0	103.4	103.3	101.1
Asterisks (*,**) denote significant difference at P≤0.05, P≤0.01 probability levels, respectively, using Fisher's least significant difference test						

Table 12. The effect of insecticides Proteus 110 OD (a.i. deltamethrin+thiacloprid 10+100 g l^{-1}), Decis 50 EW (a.i. deltamethrin 50 g l^{-1}) and Fastac EC (a.i. alpha-cypermethrin 100 g l^{-1}) on the seed yield and thousand seed weight (TSW) of spring oilseed rape in 2005 and 2006.

Parameters	Untreated	Pyrinex Supreme			Bulldock 025 EC		Proteus 110 OD
		0.75 l ha^{-1}	1.0 l ha^{-1}	1.25 l ha^{-1}	0.225 l ha^{-1}	0.3 l ha^{-1}	0.75 l ha^{-1}
	Spring oilseed rape						
	2007						
Seed yield, kg ha^{-1}	2328	2722**	2760**	2807**	2626**	2653**	2830**
% to untreated	100.0	116.9	118.6	120.6	112.8	114.0	121.6
TSW, g	3.68	3.85**	3.89**	3.90**	3.80**	3.89**	3.93**
% to untreated	100.0	104.6	105.8	105.9	103.2	105.6	106.7
	2008						
Seed yield, kg ha^{-1}	3552	3602	3546	3552	3553	3552	3552
% to untreated	100.0	101.4	99.8	100.0	100.0	100.0	100.0
TSW, g	3.84	3.91**	3.85	3.91**	3.94**	3.91**	3.90**
% to untreated	100.0	102.0	100.3	101.9	102.7	101.9	101.6
	Winter oilseed rape						
	2007						
Seed yield kg ha^{-1}	1950	2427**	2482**	2462**	2364**	2461**	2544**
% to untreated	100.0	124.4	127.3	126.2	121.2	126.2	130.5
TSW, g	4.48	4.65**	4.67**	4.67**	4.65**	4.66**	4.68**
% to untreated	100.0	103.7	104.2	104.3	103.7	104.1	104.4
	2008						
Seed yield kg ha^{-1}	5034	4771	5118	5079	5138	5110	4963
% to untreated	100.0	94.8	101.7	100.9	102.1	101.5	98.6
TSW, g	4.34	4.53**	4.56**	4.56**	4.50*	4.48*	4.51*
% to untreated	100.0	104.4	105.2	105.1	103.8	103.3	104.0
An asterisks (*,**) denotes a significant difference at P≤0.05, P≤0.01 probability levels, respectively, using Fisher's least significant difference test							

Table 13. The effect of insecticides Pyrinex Supreme (a.i. beta-cyfluthrin+chlorpyrifos 12+250 g l^{-1}), Bulldock (a.i. beta-cyfluthrin 25 g l^{-1}) and Proteus 110 OD (a.i. deltamethrin+thiacloprid 10+100 g l^{-1}) on the seed yield and thousand seed weight (TSW) of spring and winter oilseed rape in 2007 and 2008.

Parameters	Untreated	Steward EC			Mavrik 2 F
		0.0425 kg ha^{-1}	0.0625 kg ha^{-1}	0.085 kg ha^{-1}	0.3 l ha^{-1}
Spring oilseed rape					
2008					
Seed yield, kg ha^{-1}	3471	3444	3480	3396	3436
% to untreated	100.0	99.4	98.5	100.1	101.5
TSW, g	4.10	4.07	4.04	4.10	4.16
% to untreated	100.0	99.4	98.5	100.1	101.5
2009					
Seed yield, kg ha^{-1}	1936	2145	2107	2158	2108
% to untreated	100.0	110.8	108.9	111.5	108.9
TSW, g	5.68	6.08**	6.04**	5.97*	5.68
% to untreated	100.0	107.0	106.4	105.1	100.0
Winter oilseed rape					
2008					
Seed yield, kg ha^{-1}	4826	4764	4943	4891	4777
% to untreated	100.0	98.7	102.4	101.3	99.0
TSW, g	4.23	4.36**	4.35**	4.41**	4.41**
% to untreated	100.0	103.0	102.8	104.2	104.3
2009					
Seed yield, kg ha^{-1}	3942	3954	3978	4075	4017
% to untreated	100.0	100.3	100.9	103.4	101.9
TSW, g	4.54	4.55	4.59	4.62	4.59
% to untreated	100.0	100.2	101.0	101.8	101.1
Asterisks (*,**) denote significant difference at P≤0.05, P≤0.01 probability levels, respectively, using Fisher's least significant difference test					

Table 14. The effect of insecticides Steward EC (a.i. indoxacarb 300 g l^{-1}) and Mavrik 2 F (a.i. tau-fluvalinate 240 g l^{-1}) on the seed yield and thousand seed weight (TSW) of spring and winter oilseed rape in 2008 and 2009.

The efficacy of insecticides Steward EC (a.i. indoxacarb 300 g l^{-1}) and Mavrik 2 F (a.i. tau-fluvalinate 240 g l^{-1}) tested in the 2008 and 2009 cropping seasons was lower and yield increases obtained in both spring and winter oilseed rape crops were not substantial, despite the fact that in some cases substantial increase in TSW was achieved (Table 14). Our research support the findings that oilseed rape can tolerate pod infestations of < 26 % without measurable yield loss (Lerin, 1984; Buntin, 1999a).

4. Conclusions

M. aeneus was a very common and devastating pest in winter and spring rape crops every year. The research findings of the investigation on the efficacy of insecticides belonging to the different chemical classes (pyrethroids, nenicotinoids, organophosphorus and oxidiazines) for pest control in spring and winter oilseed rape suggest that all insecticides tested gave good and similar control of *M. aeneus*. Depending on the year, one or two spray applications of insecticides were used to control pollen beetle.

Our research evidenced that pod infestation by larvae of *D. brassicae*, also stem infestation by *C. pallidactylus* larvae were higher in winter oilseed rape, compared with spring rape. In general, the incidence of *C. obstrictus* infested pods was very similar in both crops, while it was variable among the crops within the same cropping season. The larvae of *D. brassicae*

were found in the assessed pods at a higher frequency, compared with *C. obstrictus*. It was estimated that the highest pod infestation by *C.obstrictus* larvae reached 6.3% in spring rape and 5.5% in winter rape, the highest pod infestation by *D. brassicae* larvae reached 8.5% and 13.4%, respectively. The number of *C. obstrictus* larvae/pod in untreated control plots was much lower, compared with the abundance of *D. brassicae* larvae. *C. pallidactylus* larvae infested 18.3-87.0% of winter rape and 0-32.5% of spring rape stems.

All tested insecticides, used for the control of *M. aeneus*, also decreased the number of pods infested by larvae of *C. obstrictus* and *D. brassicae* and decreased the number of stems, damaged by *C. pallidactylus*, in some cases these decreases were essential. The effect of different insecticides on the productivity of spring and winter oilseed rape highly depended on the cropping season and insecticide application did not always result in substantial yield increase.

5. References

Alford D V.; Nilsson C.; Ulber B. (2003). Insect pests of oilseed rape crops. In:. *Biocontrol of oilseed rape pests* Alford D. V(Ed.), pp. 9-42 ISBN 9780632054275 Oxford, United Kingdom

Barari H.; Cook SM.; Clark SJ.; Williams I.H. (2005). Effect of turnip rape (*Brassica rapa*) trap crop on stem-mining pests and their parasitoids in winter oilseed rape (*Brassica napus*). *Bio Control*, Vol 50, pp. 69-86. ISSN 1386-6141

Blight M.M.; Smart L. E. (1999). Influence of visual cues and isothiocyanate lures on capture of the pollen beetle, *Meligethes aeneus* in field traps. *Journal of Chemical Ecology,* Vol. 25, pp. 1501-1516, ISSN 0098-0331

Buntin G. D. (1999a). Damage loss assessment and control of the cabbage seed pod weevil (Coleoptera: *Curculionidae*) in winter canola using insecticides, *Journal of Economic Entomology*, Vol. 92, pp. 220–227, ISSN 0022-0493

Buntin G. D. (1999b), Cabbage seedpod weevil control and damage loss assessment in winter canola using insecticides *Proceeding of the 10th International Rapeseed Congress, ISBN* 1861660499. Canberra, Australia

Buchs W.; Katzur K. (2004): Means to control pests in organic oilseed rape production. *IOBC/wprs Bulletin,* Vol. 27 (10), pp.225-238, ISBN 978-92-9067- 235-7

Carcamo H. A.; Herle C. E.; Otani J.; McGinn S. M. (2009). Cold hardiness and overwintering survival of the cabbage seedpod weevil, *Ceutorhynchus obstrictus. Entomologia Experimentalis et Applicata*, Vol. 133, pp. 223–231, ISSN 0013-8703

Cook S. M.; Watts N. P.; Hunter F.; Smart L.E.; Williams I.H. (2004). Effects of a turnip rape trap crop on the spatial distribution of *Meligethes aeneus* and *Ceutorhynchus assimilis* in oilseed rape. *IOBC/wprs Bulletin Vol*. 27(10) pp. 199-206, ISBN 978-92-9067- 235-7

Cook S.M.; Smart L. E.; Martin J. L.; Murray D. A.; Watts N. P.; Williams I. H. (2006) Exploitation of host plant preferences in pest management strategies for oilseed rape (*Brassica napus*). *Entomologia Experimentalis et Applicata,* Vol. 119 pp. 221-229, ISSN 0013-8703

Dechert G,; Ulber B. (2004). Interactions between the stem-mining weevils *Ceutorhynchus napi* (Gyll.) and *Ceutorhynchus pallidactylus* (Marsh.) (Coleoptera: *Curculionidae*) in oilseed rape. *Agricultural and Forest Entomology,* Vol. 6, pp. 93–198, ISSN 1461-9563.

Decoin M. (2002). The rape blossom beetle affair. Focus on insecticide resistance: study, understand and advise. *Phytoma,* Vol. 555, pp. 24–26, ISSN: 0048-4091

Derron J.O., Clech E., Bezencon I.; Goy G. (2004). Resistance of the pollen beetles (*Meligethes* spp.) to pyrethroids in western Switzerland. Revue Suisse d'Agriculture Vol. 36, No. 6, pp. 237-242, ISSN 03751325

Dosdall L.M. (2009). Responses of the cabbage seedpod weevil, *Ceutorhynchus obstrictus* (Marsham) (Coleoptera: *Curculionidae*), to seed treatments of canola (*Brassica napus* L.) with the neonicotinoid compounds clothianidin and imidacloprid. *Pest Management Science*, Vol. 65, pp. 1329-1336, ISSN 1526-4998

Eickermann M. (2008). Effect of *Brassica* genotype on the infestation by cabbage stem weevil *Ceutorhynchus pallidactylus* (Mrsh.) (Col.: Curculionidae) and the parasitism of stem weevil larvae. Dissertation. German

Garthwaite D. G.; Thomas M. R.; Hunt M. (1995). Pesticide usage survey - arable farm crops 1994. *Report 127, Ministry of Agriculture, Fisheries and Food*, London, United Kingdom

Hansen L. M. (2003a). Insecticide-resistant pollen beetles in Danish oilseed rape // *Pest Management Science*. Vol. 59 (9), pp. 1057–1059, ISSN 1526-4998

Hansen L. M. (2003b). A model for determination of the number of pollen beetles (*Meligethes aeneu*s F.) per plant in oil-seed rape crops (*Brassica napu*s L.) by estimating the percentage of plants attacked by pollen beetles. *Journal of Applied Entomology*, Vol. 127, pp. 163–166, ISSN: 0931-2048

Hansen L. M. (2008).Occurrence of insecticide resistant pollen beetles (*Meligethes aeneus* F.) in Danish oilseed rape (*Brassica napus* L.) crops. *OEPP/EPPO Bulletin,* Vol. 38 , pp. 95–98 ISSN: 1365-2338

Heimbach U.; Muler A.; Thieme T. (2007). First Steps to analyse pyrethroid resistance of different oilseed rape pests in Germany. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes,* Vol. 58 pp. 1-5, ISSN 0027-7479

Henderson, C. F., Tilton E. W. (1955). Tests with acaricides against the brow wheat mite. *Journal of Economic Entomology*, Vol. 48, pp. 157-161, ISSN 0022-0493

Kazachkova N.I. (2007). Genotype analysis and studies of pyrethroid resistance of the oilseed rape (Brassica napus) Insect pest pollen beetle (*Meligethes aeneus*). *Thesis of Swedish University of Agriculture Sciences*, pp. 1-20, ISBN 978-91-576-7310-7, Upsala, Sweden

Kirch G. (2006). Auftreten und Bekämpfung phytophager Insekten an Getreide und Raps in Schleswig-Holstein. *Inaugural-Dissertation zur Erlangung des Doktorgrades,* pp. 11-37 Giessen, German

Lamb R.J. (1989). Entomology of oilseed *Brassica* crops. *Annual Review of Entomology*, Vol. 43, pp. 211-229, ISSN 0066-4170

Landschreiber M (2005). Die Vorblütenschädlinge sind weiter auf dem Vormarsch. *Raps*, Vol 23, pp. 4-9

Lancashire P.D., Bleiholder H., Van dem Boom T., Langeluddecke P., Staugs R., Weer E., Witzenber A. (1991). A uniform decimal code for growth stages of crops and weeds. *Annuals Applied Biology*, Vol 119, pp. 561–601, issn 1744-7348

Lerin, J. (1984). Assessment of yield loss in winter rape due to seed-pod weevil (*Ceutorhynchus assimilis* Payk.) II. Yield loss in a cage experiment. *Agronomie 4*, pp. 147-154.

Lithuanian department of statistics, http://db1.stat.gov.lt/statbank/default.asp?w=1280 Acssess in : 07 18 2011.

Milovac Z.; Keresi T.; Mitrovic P.; Martinkovic R.; Martinkovic- Jeromela A. (2011). Incidence of the Cabbage Seedpod Weevil (*Ceutorhynchus obstrictus)* and the brassica pod midge *(Dasineura brassicae)* in rapeseed in Vojvodina province. *Proceeding of the 13th*

Murchie A.K.; Smart L.E.; Willims I.H; (1997). Responses of *Dasineura brassicae* and its parasitoids platygaster subuliformis and omphale clypealis to field traps baited with organic isothiocyanates. *Journal of Chemical Ecology*, Vol. 23 (4), pp. 917-926 ISSN 0098-0331

Nuss H.; Ulber B. (2007). Effect of sowing rate on the infestation of winter oilseed rape by stem-boring pests and on yields. *12th International Rapeseed Congress*, pp. 290-293 ISBN 1-933100-20-6 Wuhan, China

Pavela R.; Kazda J.; Herda G. (2007): Influence of application term on effectiveness of some insecticides against brassica pod midge (*Dasineura brassicae* Winn.). Plant Protection Sciences, Vol. 43, pp. 57–62, ISSN 0971-3573.

Pavela R.; Kazda J.; Herda G. (2009). Effectiveness of Neem (*Azadirachta indica*) insecticides against Brassica pod midge (*Dasineura brassicae* Winn.). *Journal of Pest Science*, Vol. 82, No. 3, pp. 235-240, ISSN 1612-4758

Petraitiene E.; Brazauskiene I.; Smatas R.; Makunas V. (2008). The spread of pollen beetles (*meligethes aeneus*) in spring oilseed rape (*brassica napus*) and the efficacy of pyrethroids *Zemdirbyste-Agriculture*, Vol. 95, No. 3 pp. 344–352, ISSN 1392-3196

Ruther J.; Thiemann K. (1997) Response of the pollen beetle *Meligethes aeneus* to volatiles emitted by intact plants and conspecifics. *Entmologia Experimentalis et Applicata*, Vol. 84, pp. 183-188, ISSN 0013-8703

Tamutis V. (1997). Fauna and biology of weevils (*Coleoptera, Curculionidae, Ceutorhynchus*), and their role in agrocenosis of winter rape. Dissertation. Kaunas

Williams I. H. (2004). Advances in insect pest management of oilseed rape in Europe. In: *Insect pest management: field and protected crops* Horowitz A. R.; Ishaaya I. (eds.), pp. 181–208, ISBN 3-540-20755-4 Berlin, New York

Williams I. H.(2010) The major insect pests of oilseed rape inEurope and their management: an overview. In. *Biocontrol-based integrated management of oilseed rape pests*, Williams I. H. (Ed.), pp. 1-45, ISBN 978-80-481-3982-8, London, New York,.

Wegorek P. (2005). Preliminary data on resistance appearance of Pollen beetle PB (*Meligethes aeneu*s F.) to selected pyrethroids, organophosphorous and chloronicotynyls insecticide, in 2004 year in Poland. *Resistant Pest Management Newsletter*, Vol. 14 (2), pp. 19–21

Wegorek P.; Obrepalska-Steplowska A.; Zamoyska J.; Nowaczyk J.(2006): Resistance of pollen beetle (*Meligethes aeneus* F.) in Poland. *Resistant Pest Management Newsletter*, Vol. 16, pp. 28-29

Wegorek P.; Mrowczynski M.; Zamojska J. (2009). Resistance of pollen beetle (*Meligethes aeneus* F.) to selected active substances of insecticides in Poland. *Journal of Plant Protection Research*, Vol.49, No. 1, pp. 119-127, ISSN 1427-4345

Veromann E.; Kevvai R.; Luik A.; Williams I. H. (2008). Do cropping system and insecticide use in spring oilseed rape affect the abundance of pollen beetles (Meligethes aeneus Fab.) on the crop? *International Journal of Pest Management*, Vol. 54 No. 1, pp. 1 – 4, ISSN 0967-0874

16

Secondary Metabolism as a Measurement of Efficacy of Botanical Extracts: The Use of *Azadirachta indica* (Neem) as a Model

Moacir Rossi Forim, Maria Fátima das Graças Fernandes da Silva and João Batista Fernandes
Universidade Federal de São Carlos
Brazil

1. Introduction

This chapter is primarily concerned with the concept and correct use of natural products as insecticides. All organisms need to transform and interconvert a vast number of organic compounds in order to be able to live, grow, and reproduce. They need to provide themselves with energy in the form of ATP and a supply of building blocks to construct their own tissues. Despite the extremely varied characteristics of living organisms, the pathways for generally modifying and synthesizing carbohydrates, proteins, fats, and nucleic acids are found to be essentially the same in all organisms, apart from minor variations. These processes demonstrate the fundamental unit of all ways of life, and are collectively described as primary metabolism. In contrast to these metabolic pathways, which synthesize, degrade, and generally interconvert compounds commonly encountered in all organisms, there is also an area of metabolism concerned with compounds which have a much more limited distribution in nature called secondary metabolism (Dewick, 2009).

Secondary metabolites or natural products are commonly reserved for organic compounds of natural origin that are unique to one organism, or common to a small number of closely related organisms (Mann, 2005). Secondary metabolites are not necessarily produced under all conditions, and in the vast majority of cases the function of these compounds and their benefit to the organism are not yet known. In fact, they are usually an expression of the individuality of species (Dewick, 2009). In most examples they appear to be non-essential to the plant, insect, or microorganism producing them. For instance, the morphine only occurs in two species of poppy, *Papaver somniferum* and *P. setigerum*, and although it is widely used and abused by man, it has no function which is known in these plants (Mann, 2005).

Plants produce a large diversity of natural products which are usually sub-divided in classes according to metabolic pathways at polyketides, lignans, coumarins, flavonoids, terpenoids, steroids, alkaloids, *etc*. These are of great importance for the plant for their interaction with the environment due to their roles as pollinator attractants, for symbiosis and for defense against attacks by microorganisms, other plants or animals. Moreover, they are economically important to man as a source of pharmaceuticals, flavours, fragrances, insecticides, dyes, food additives, toxins, *etc*. (Zarate et al., 2010). Structures of an estimated 200,000 natural products have been elucidated (Dixon & Strack, 2003). Nowadays,

researches for new insecticides are a promising area which is showing the growing demand for natural products. The diversity of secondary metabolites from plants may be commercially explored as botanical extracts or pure compounds after extraction and isolation by phytochemical process.

2. Natural products as insecticides

Insecticides are the cornerstone upon which the pest management practices are based, and are likely to remain so as long as affective and inexpensive chemicals are available (Hayves, 1988). Natural products have been used as botanical pesticides since ancient times. Apparently, almost every plant species has developed a unique chemical complex that protects itself from pests (allelochemicals). Thus, plants offer us a diverse group of complex chemical structures and almost every imaginable biological activity. For thousands of years, agricultural practices relied heavily on crop rotation or mixed crop planting to optimize natural pest control (such as predation, parasitism, and competition). Therefore, the concept of 'natural pesticides' arose early along with the development of agriculture (Dayan et al., 2009). The medical compendium known as the Ebers Papyrus of *c.* 1600 B.C. includes both chemical and organic substances recommended as insecticides (Panagiotakopulu et al., 1995). In the first century AD, Pliny the Elder, the Greek philosopher, wrote ''Natural History'' in which he recorded all the known pest control methods. At the same time the Chinese recorded their use of powdered chrysanthemum as an insecticide. Methods such as mulching and burning, as well as the use of oils for pest control were mentioned. A survey of the Shengnong Ben Tsao Jing era (25–220 A.D.) shows that 267 plant species were known to have pesticidal activity (Dayan et al., 2009; Yang &Tang, 1988).

In fact, chemistry has always fulfilled an important role with the introduction of a lot of essential products to humanity. These improvements are easily seen in food production. In the last 50 years, farmers around the world have trusted substantially in the use of fertilizers and protecting organic-synthetic pesticides, therefore improving each year their yields of production and supplying the world demand of foods and natural fibers (Knowles, 2008). In this period, the synthetic pesticides have been the main insecticide tool (Saxena, 1989). Chemical industry has been assisting the demand of consumers, which increased the gains in crops through continuous development and introduction of new synthetic products. However, not only have the yield in crops increased, but also the world population, which causes a constant pressure to improve such a production (Knowles, 2008).

Although the use of synthetic insecticides have been efficient to control insect pests, their extensive and sometimes indiscriminate use have caused various problems of social and environmental repercussion (Alkofani et al., 1989). As negative consequences of using these products are countless and cumulative damages to environment. Among them there is both the contamination of soil, air, waters, fishes, animals, and the man himself (as much in fields as in consumption of contaminated products) and the reduction of biodiversity, population of natural enemies, pollinators and bees. Such reductions allow secondary pests to appear. Furthermore, the indiscriminate use of synthetic insecticides has allowed insects to develop resistance against them (Dayan et al., 2009; Jadeja et al., 2011). Concern about the adverse impacts of pesticides on the environment and on human health started to be voiced in the early 1960s (Carson, 1962). Since then, debate on the risks and benefits of pesticides has not ceased and a huge amount of research has been conducted into the impact of pesticides on the environment (van der Werf, 1996).

Along with the evolution of knowledge about environment damages, the natural-products concept has come back to the worldwide scenario as a proposal for alternative pest control agents but with reduced environmental consequences, which have been creating or causing an evolution in several research lines. In fact, prior to the discovery of the organochlorine and organophosphate insecticides in the late 1930s and early 1940s, botanical insecticides were important products for pest management (Isman, 1997).

Natural products represent a rich source of biologically active compounds and are an example of molecular diversity, with recognized potential in pesticides discovery and development. They are in general structurally more complex, selective and biodegradable, environmentally compatible and less toxic to non-target organisms than synthetic pesticides (Alkofani et al., 1989; Duke et al., 2000; Rattan, 2010). Each molecule present in the secondary metabolism of plants may be explored individually either as a model to the development of new insecticides or as an isolated molecule which is an active compound of new insecticides. Their chemical diversity, resulted from effects of evolutionary pressure to create biologically active molecules, is similarly structured to protein targets of many species, showing several biological activities (Harvey, 2007). Moreover, plants usually feature synergism, which is a combination of effects equal to the sum of those of individual components, or it takes place when combinations of bioactive substances exceed in effects that are greater than the sum of those of individual components (Schmidt, 2008). This last feature describes several important properties of the use of botanical extracts. The mixture of secondary metabolites may be deterrent to insects for a longer period than single compounds and, different physical properties may allow more deployment or longer persistence of defenses (Rattan, 2010). Thus, it is necessary that technical and economical studies evaluating if it is better to work by using a single molecule or botanical extracts are developed.

Compounds of botanical extracts, in many cases, have the function of protecting the active molecules against alterations, namely oxidations, hydrolyses, or other, and they are able to allow better absorption by target organisms facilitating the transport through membranes or inhibit enzymatic systems. Furthermore, insects tend to acquire resistance against formulated active compounds, which is harder to occur by using botanical extracts. For instance, Feng & Isman (1995) show that *Myzus persicae* acquired in few generations resistance against azadirachtin, an isolated secondary metabolite. However, this species did not show any resistance during forty generations by using botanical extracts of *Azadirachta indica*, the main source of azadirachtin. Finally, botanical extracts are cheaper than both the development of new synthetic compounds and isolation processes of secondary metabolites (Duke et al., 2000).

3. Phytochemical sources and insecticidal activity

The growing demand for natural products has been intensified in the past decades as they are extensively used as biologically active compounds and are being considered an important alternative strategy for sustainable insect pest management in agriculture, because they are biodegradable and potentially suitable for the use in integrated management programs (Rattan, 2010). A brief research into the literature reveals many investigations applied into the biological activity of many plant components against a large number of pathogens and arthropods. An old review with an agricultural focus by Roark (1947) described around 1200 plant species that have been listed in the literature as

having potential insecticidal value. These studies have exposed an array of botanical insecticides in several families such as Meliaceae, Agavaceae, Lamiaceae, Rutaceae, Cactaceae, Asteracae, Labiatae, *etc*., containing a wide spectrum of bioactive fungicides, nematicides, acaricides, insecticides and carcinogenic (Shaalan et al., 2005). Some of the botanical extracts of insecticidal interest are described in Table 1. It is possible to identify extracts prepared from roots, stems, branches, fruits, seeds, leaves, flowers, *etc*. of plants, which show different biological activities against insect pests (different ways of action), a large diversity of phytochemical techniques employed during the production of these materials, variation in the chemical profile and various compositions in formulations which were assayed.

Plant	Organs	Insect	Source
Aglaia odorata	leaves	*Spodoptera littoralis*	Nugroho et al., 1999
Yucca periculosa	barks	*Spodoptera frugiperda*	Torres et al., 2003
		Sitophilus oryzae	
		Tribolium castaneum	
Ocimum gratissimum	oils	*Oryzaephilus urinamensis*	Ogendo et al., 2008
		Rhyzopertha dominica	
		Callosobruchus chinensis	
Evodia rutaecarpa	essential oil	*Sitophilus zeamais*	Liu & Ho, 1999
		Triboliumcastaneum	
Azadirachta indica	seeds, metabolites, oils	*Hypsipyla grandella*	Mancebo et al., 2002
		Plodia interpunctella	Rharrabe et al., 2008
		Callosobruchus maculatus	Lale & Abdulrahman, 1999
		Plutella xylostella	
Artemisia scoparia	essential oils	*Callosobruchus maculatus*	Negahban et al.,2006
		Sitophilus oryzae	
		Tribolium castaneum	
Dysoxylum malabaricum	leaves	*Anopheles stephensi*	Nathan et al., 2006a
Melia azedarach	leaves, seeds	*Hyblaea puera,*	Nathan et al., 2006b
		Plutella xylostella	
Ocimum basilicum	essential oils	*Callosobruchus maculatus*	Kéita et al., 2001
Roldana barba-johannis	aerial parts	*Spodoptera frugiperda*	Céspedes et al., 2004
Myrtillocactus geometrizans	roots, aerial parts	*Spodoptera frugiperda*	Céspedes et al., 2005
		Tenebrio molitor	

Table 1. Botanical extracts and their potential use against insect pests.

In fact, it is not so hard to identify botanical extracts which show some kind of activity against insect pests. In the universe of plants, the Meliaceae family has drawn attention. This family comprises 50 genera and 1400 species, mostly distributed in the pantropical zone. Among the genera, the ones which show greater insecticidal activities are *Aglaia, Aphanamixis, Azadirachta, Garapa, Cedrela, Chukrasia, Dysoxylum, Guarea, Khaya, Melia, Soymida, Swietenia, Trichilia, etc*. Most of the plants of this family are trees and are well known for their quality timber (Benerji &Nigam, 1984).

Taking *Azadirachta indica* A. Juss (Meliaceae) (Neem tree) as a highlight, researches have depicted various mechanisms of action for its insect control. More than 100 compounds have been isolated from various parts of the Neem (Luo et al., 1999). It is common to find a range of biological activities, including insect anti-feedant and growth regulating properties, anti-bacterial, anti-fungal and anti-viral activities, anti-protozoal, and anti-sickling properties. In the recent past, chemical constituents of Neem seeds have been intensively explored since they have proved to be an excellent source of a wide variety of chemicals useful to the management of pestiferous insects (Kumar et al., 2003). More than 500 insect pest species are listed as sensitive to Neem seed extracts (Morgan, 2009). These biological proprieties are caused mainly by terpenoid compounds several papers are reported to contain bitter substances, popularly known as limonoids (Figure 1) (Luo et al., 1999; Siddiqui et al., 1999; Siddiqui et al., 2001). The main metabolite of Neem is a limonoid known as azadirachtin (Figure 1).

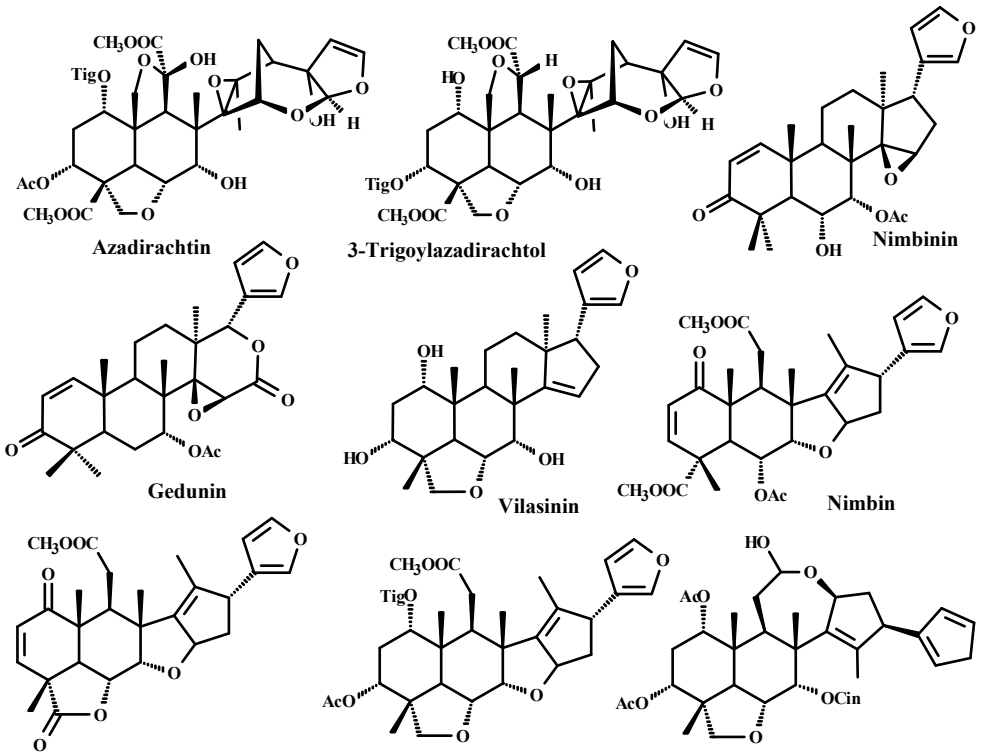


Fig. 1. Main limonoids isolated from species *Azadirachta indica*.

These substances are important enzymatic and metabolic inhibitors. Apart from efficacy and spectrum-of-action described to Neem, its biological criteria still include favorable toxicology and minimal environmental impacts (*i.e.*, vertebrate selectivity; selectivity favoring natural enemies and pollinators; and rapid environmental degradation) (Isman, 1997). Some biological activities by Neem are described in Table 2.

Activity	Botanical extracts	Target species	Source
Acaricidy	Extracts of neem oil	*Sarcoptes scabiei larvae*	Du et al., 2008
Parasiticide	Seed oil	*Muscidifurax raptor*	Ruiu et al., 2008
Antifeedant	Extracts of the seeds	*Schistocerca gregaria*	Butterworth & Morgan, 1971
Endocrine control	Azadirachtin	*Locusta migratoria*	Sieber & Rembol, 1983
Control of pests	Extracts of the leaves	*Spodoptera frugiperda* *Macrodactylus spp.* *Frankliniella spp.*	Montes-Molina et al., 2008
Nematicidy	Extracts of the leaves and cake	*Meloidogyne javanica*	Javed et al., 2008
Growth inhibitor	azadiradione	*Heliothis virescens*	Lee et al., 1988
Antifeedant, Disruptor of insect development, Effective sterilant	Azadirachtin, Azadirachtin-containing extracts	*Schistocerca gregária* *Phormia terrae-novae* *Leptinotarsa decemlineata* *Oncopeltus fnsciatus*	Schmutterer, 1988
Mortality, Control of weight	Extracts of seed kernels	*Nilaparvata lugens*	Nathan et al., 2007
Effects on survival, Fecundity, Development, Oviposition, Feeding	Seed oil	*Plutella xylostella*	Charleston et al., 2006
Insecticide	Seed kernels	*Cnaphalocrocis medinalis*	Nathan et al., 2006c
Oviposition, Larval development, Feeding	Seed oils	*Mamestra brassicae*	Seljasen et al., 2006
Antifeedant	Neem oil	*Hylobius abietis*	Thacker e tal., 2004

Table 2. Different activities identified of *Azadirachta indica* to control several insect pests.

4. Problems associated to the use of natural products

Despite the favorable characteristics of the use of botanical extracts above-mentioned, as well as the large availability of vegetal species, and the huge quantity of works carried out proving their biological efficacy, few products have been commercially available lately. It is possible to identify some limitations inherent to their success though. Problems as low production, regulation by Federal Institutions, difficulty during application, storage, stability, quali and quantitative reproducibility of secondary metabolites, repeatability of biological activity, *etc.*, need to be approached before the botanical extracts are acquired safely (Isman, 1997).

The comprehension of these problems perhaps helps in the understanding of why various authors have described different results of tested insecticidal activity by using botanical extracts of the same species, and at the same time, to learn their efficient manipulation and

correct use. How is it possible for botanical extracts prepared from the same vegetal species to show different results towards a target insect? For instance, Roel et al. (2010) describe a lethal action against the larvae of *Spodoptera frugiperda* by using formulations with 0.4% (w/v) of the Neem oil. However, Viana & Plates (2003) relate a dose of 1.0% (w/v) of aqueous extract of the Neem leaves to obtaining the same action, which was 2.5 times higher concentration than the one related by Roel et al.

Biological activities of botanical extracts come from secondary metabolites, which are present in these materials. Several factors may change the stability of products or active compounds of natural source. Each component, active or not, present in different quantities, may affect the stability of the products. Other factors known as extrinsic such as temperature, radiation, light, air (especially oxygen, carbon dioxide, and steam of water), humidity, seasonality, place and hour of the collection, storage, *etc*., may change the stability and quantity of an active compound or botanical extract (Gobbo-Neto & Lopes, 2007). For example, contend of hypericin and pseudo-hypericin in *Hypericum perforatum* nearly increase 30 times in the summer (Southwell & Bourke, 2001). Concentration of biflavones as ginkgetin in *Ginkgo biloba* leaves also shows seasonal changes (Lobstein et al., 1991). Wallaart et al. (2000) described the reduction of biosynthetic precursor dihydroartemisinic acid happening concomitantly with the production of artemisinin in *Artemisia annua* after metabolic stress caused by low temperature. In *Hypericum perforatum* flowers a significantly rise in concentration of flavonoids, hypericins and chlorogenic acid occur under hydric stress along with the reduction in hyperforin content (Gay et al., 2003). Higher production of phenolic compounds as flavonoids, tannins, anthocyanins, *etc*. are usually observed in plants under high solar radiation. Such a factor has also affected other secondary metabolic classes such as alkaloids and terpenoids (Gobbo-Neto & Lopes, 2007). Also, there are the intrinsic factors such as incompatibility, pH, hydrolyzes, racemization, and oxidation (Barrek et al., 2004).

For example, a rapid photodecomposition of azadirachtin, the main metabolite found in extracts of seeds from Neem, has been observed with spray applications onto conifer and deciduous foliage. Experimental studies showed a dissipation half-life (DT_{50}) to azadirachtin of about 20 h (Sundaram & Curry, 1994). Its short environmental persistence is due to the presence of sensitive moieties such as *p*-electrons, ester linkages, furan, and an epoxide ring (Gopalakrishnan et al., 2001; Wei-Hong & Zhan-qian, 2006). However, the major problem is its sensitivity to photodegradation, therefore it is rapidly lost in sunlight. Through a simple experiment carried out, a pure sample of azadirachtin was solubilized in a mixture of water:ethanol (4:1) and exposed to light, at ambient temperature, during seven days. In the end, a mixture of sub-products of degradation was obtained, considering that the three most abundant sub-products were identified by spectroscopy and spectrometric techniques. These sub-products are shown in Figure 2. Sub-product 3 was isolated and its structure determined in literature, by Kumar et al. (1996), as 1-tigloil-3-acetilazadirachtinin. It is important to highlight that this product was firstly identified as a natural product, however, it could solely have been a product of degradation. These examples show low stability for molecules of botanical extracts as azadirachtin. In this case, it is necessary that new formulations or systems of protection for the insurance and efficient use in field are developed.

Another situation is the degradation of extracts, which happens in commercial products, for instance in this case of Neem (oils of seed kernels). This degradation may occur even when it is stored under appropriate conditions, light shelter, temperature and humidity. In another

work, by using chromatography techniques, it was possible to observe the degradation of azadirachtin in commercial oil of Neem. Samples of Neem oil were left to rest in a dark chamber at 20°C. At specific times, the samples were homogenized and a fraction of those was analyzed. Figure 3 shows the relation between the quantity of azadirachtin and the time. It is easy to observe that the quantity of azadirachtin in these products was gradually decreased during the time of storage. These results are extremely alarming. Considering that azadirachtin has been the most potent secondary metabolite of Neem, its degradation may compromise the efficiency of commercial products (Nathan et al., 2006c).

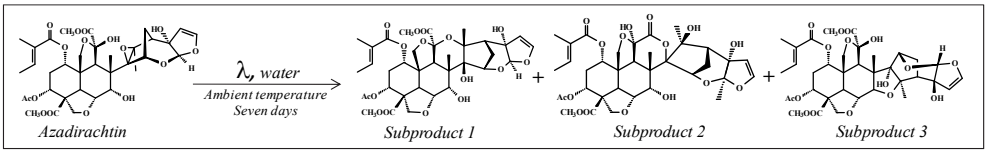


Fig. 2. Identified products of degraded azadirachtin.

Hypothetically, when farmers buy a natural pesticide as Neem oil, in fact they might be buying a product with approximately 1,000 µg kg^{-1} or with 200 µg kg^{-1} containing azadirachtin as standard. Obviously, the results and efficiency in field will be very different. In this case, the efficiency depends on the quantitative and manufacturing data of both examples of Neem oil.

These problems may create a generalized discredit about the use of natural products such as Neem. A not very well-informed consumer may use Neem oil sold after a long period of storage and not have the expected action in field. This farmer will probably not trust in the use of this kind of product anymore. As in any other commercial product, botanical extracts need to be subjected to and monitored by control quality programs.

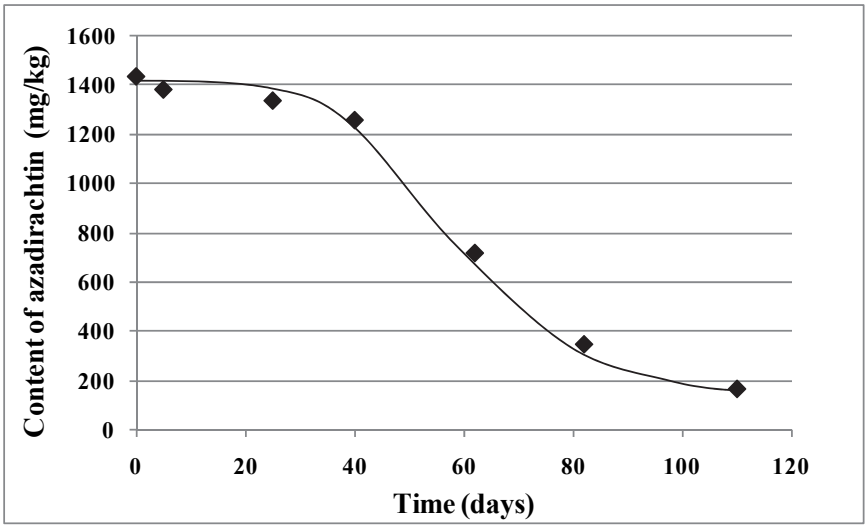


Fig. 3. Degradation curve of azadirachtin to commercial Neem oil in storage.

5. Quality control on natural products

The potential of natural products as agents to control insect pests is clear in several papers which describe their biological activities (Céspedes et al., 2004, 2005; Charleston et al., 2006; Nathan et al., 2006a, 2006b; Negahban et al.,2006; Rharrabe et al., 2008; Torres et al., 2003). However, in order for the use of natural products to thrive, as an alternative to traditional synthetic pesticides, some parameters of production and quality control should be observed, especially a) seasonal variation; b) formulation and stability and c) development of methods to quality control. These concerns introduce the importance of quality control to botanical extracts, which are desirable to be used as the new source of natural pesticides.

Naturally, the quali and quantitative composition of secondary metabolites in a botanical extracts vary according to how plants are affected by genetics, ontogenesis and environment factors in which they are cultivated (Arimura et al., 2005). In studies carried out by Forim et al. (2010a) using seed kernels of Neem from various Brazilian regions analyzing the content of azadirachtin e 3-tigloylazadirachtol by HPLC, two of the main limonoids responsible for the insecticidal activity of Neem (Sidhu et al., 2003), it varies from 1,516.4 to 5,117.1 mg kg^{-1} and from 224.7 to 1,116.5 mg kg^{-1}, respectively (Forim et al. 2010a).

Genetics and seasonal variations are not easy to control. However, the content of quality markers to botanical extracts, such as azadirachtin (Figure 1) to Neem extracts should be monitored. The quantitative knowledge of these markers allows commercial products to be prepared by reproducing the amount of such markers and, consequently, the biological efficacy of these products.

In general, one or two markers of the active components in botanical extracts are currently employed to evaluate their quality and authenticity. This kind of analysis, however, does not give a complete profile of a botanical product because multiple constituents are usually responsible for its pesticide effects. These multiple constituents may work "synergistically" and can hardly be separated into active parts. Thus, it should be necessary to determine most of the phytochemical constituents in order to ensure the reliability and repeatability of the botanical pesticide effect.

Considering the large diversity of compounds present in one botanical extract, several, slow and expensive chromatographic techniques, such as high-performance liquid chromatography (HPLC), gas chromatography (GC), capillary electrophoresis (CE), *etc*. should be applied to this kind of documentation (Liang et al., 2004). In addition to these techniques, the following technological revolution which had a tremendous impact upon the analysis of natural products was the development of detectors such as electrospray ionization mass spectrometry (ESI-MS) and nuclear magnetic resonance (NMR). These detectors may be utilized online combined with chromatography techniques as a powerful complement to HPLC detection system (Strege, 1999). Through the use of these techniques, along with appropriate procedures of sample preparation, it is possible to develop methods of high-throughput screening identifying and quantifying botanical extracts. However, these methods are expensive for routine use.

On the other hand, through the use of markers it is possible to simplify the demand of equipment, time and the cost of analyses, which make possible the use of simple analytical methods during the development of new botanical products. As a matter of fact, this procedure should be a routine practice. In general, simple HPLC equipment is necessary. When coupled with phododiode-array UV-Vis absorbence detection, HPLC serves as a powerful tool for the rapid characterization of natural product extracts (Strege, 1999).

The step of sample preparation for the analysis of constituents present in botanical extracts is as important as choosing the analytical instrumentation. This procedure may be selective or specific to botanical markers, or not, when the aim is a full characterization of the extract. This step involves two parts: extraction and pre-treatment phases. The main methods of botanical extracts preparation are sonication, soxlet extraction, microwave assisted extraction, supercritical fluid extraction, accelerated solvent extraction, pressurized hot water extractions, *etc*., by using solvents as methanol, ethanol, water, a mixture of them, carbon dioxide, *etc.* (Ong, 2004). Pre-treatment steps may be performed by using solid-phase extraction, solid-phase microextraction, matrix solid-phase dispersion, filtration, dilution techniques, *etc.* (Rijke et al., 2006). It is very important to be sure that in extracting procedures the extraction efficiency is as high as possible, and that during the pre-treatment steps there is nether loss nor degradation of analytes.

6. Methods of Neem quality control

In the case of Neem, azadirachtin, previously known as azadirachtin A, is a good quality marker. The amount of this limonoid may be easily determined in commercial products, organic extracts, seed kernels and cakes by using HPLC (Forim et al., 2010a). In our laboratory, we have been using a HPLC of Agilent 1200 Series Liquid Chromatography apparatus (Agilent Technologies, Santa Clara, USA), configured with a degasser G1322A, quaternary pump G1311A, autosampler G1329A, column oven G1316A and a simple UV detector G1314B. The degasser and quaternary pump are optional, which may be replaced by a simple pump simplifying the instrumental demand. Chromatography run is a reversed-phase procedure utilizing a stainless steel Zorbax Eclipse XDB®C18 column (150x4,6 mm i.d., 5µm particle size, Agilent, USA) fitted with a Phenomenex® C18 (4x3mm i.d., 5µm particle size, Torrance, CA, USA) security guard cartridge. The control of the HPLC system, acquisition and processing of the data collection are realized by Agilent Technologies EZCrom SI software (G6702AA).

The chromatographic analyses were performed in isocratic mode. The mobile phase consists of acetonitrile and water (35:65, v/v). The column temperature is maintained at 30°C. The flow rate is 1.0 ml min^{-1} with an injection volume of 10 µl. All experiments are performed at 217 nm. This wavelength was selected because it is a UV maximum and provides the sensitivity needed for the quantification of the low markers concentration in the samples (Forim et al., 2010a). Other analytical methods applied to the quality control of botanical extracts of Neem have been described by Sharma et al. (2003) by using HPLC-PDA under isocratic conditions; Shidu et al. (2003) by using HPLC-UV under gradient conditions; Thejavathi et al. (1995) by using HPLC-UV under gradient conditions and work with internal standard, *etc*. These methods have been successively well employed in quality control of Neem products.

It is important to highlight that all analytical methods needed to be previously validated in order to be reliable. HPLC methods usually use parameters of validation such as linearity, specificity, accuracy, precision, robustness, recovery, limits of quantification (LOQ) and detection (LOD), and repeatability (ICH, 1996).

A good linearity to the analysis of Neem products was found from 1 to 70 µg ml^{-1}; the limits of detection and quantification were smaller than 1.0 and 0.3 µg ml^{-1}, respectively; the precision and accuracy were inferior to 3%. Azadirachtin and 3-tigloylazadirachtol

showed a separation factor (α) of 1.10 and the resolution (R_s) was 2.09 between themselves (Forim et al., 2010a). Figure 4 shows a chromatogram of the analysis of azadirachtin and its selectivity between 3-tigloylazadirachtol in methanolic extract of seed kernels from *Azadirachta indica*. As important as developing and validating a new analytical method is to identify an efficient procedure to prepare the samples. Extracts of seed kernels or Neem oil undergo a process of clean up by using cyano solid extraction phases columns before HPLC analyses.

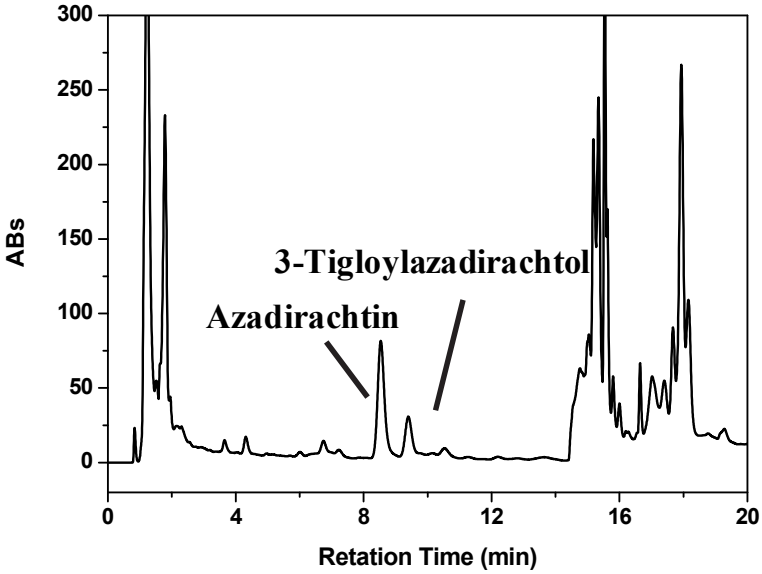


Fig. 4. Standard chromatogram of analysis of *Azadirachta indica* products.

7. Biological activity of Neem and analytical methods

Methods as above described are useful to control the quality of botanical extracts and help in the connection of these products with their botanical assays and the desired insecticidal activity. Forim et al. (2010b) published a work in which they use this HPLC method to evaluate the biological activity of Neem extracts. In this work several Neem extracts were obtained from different ways and assayed against *Spodoptera frugiperda,* an important maize pest. All botanical extracts were prepared by using the same seed kernels (lot). In this case, changes in genetics, ontogenesis and environment factors did not occur among Neem extracts prepared. The only change was in the way of preparing the extracts.

Techniques of extraction were: a) maceration of mill seed kernels by using two solvents at ambient temperature: firstly, maceration by using *n*-hexane (5 x 12 hours) which created a non-polar extract and a cake, and secondly by using ethanol (5 x 12 hours) to extract more polar compounds of the cake (**MHE**); b) maceration by using methanol (5 x 12 hours) at ambient temperature (**MM**); c) maceration by using ethanol (5 x 12 hours) at ambient temperature (**ME**); d) maceration by using ethanol under constant agitation (5 x 12 hours) at ambient temperature (**MEA**); e) extraction by ethanol using ultra-son (5 x 10 minutes) at

ambient temperature (**EU**); f) extraction by ethanol using centrifugation (5 x 10 minutes) at ambient temperature (**EC**); and g) maceration by ethanol under simple agitation (5 x 10 minutes) at ambient temperature (**MV**). At the end, all solvents were evaporated by using rota-evaporator, producing dry botanical extracts which were assayed against *Spodoptera frugiperda.*

Through the HPLC analytical method for Neem previously described, it was possible to determine the quantity of this marker in the seed kernels utilized in the extraction processes, and in the final Neem extracts, which made the calculations of extraction efficiency possible. The amount of azadirachtin in each extract and the extraction efficiency of each process are described in Table 3.

Extract	Extraction Efficiency (%)	Amount of azadirachtin (mg kg^{-1})
MHE	100.1	32,480.3
MM	54.3	12,070.8
ME	100.1	21,046.9
MEA	99.1	19,534.8
EU	56.6	29,464.6
EC	45.1	1,385.0
MV	58.0	18,459.2

[a] Relative Standard Deviations among extracts were smaller than 12% (n = 3); [b] Initial amount of azadirachtin in seed kernels was 2,348.5 mg kg^{-1}.

Table 3. Amount of azadirachtin in Neem extracts, which were assayed against *S. frugiperda*.

Naturally, each process showed individual yield in both the extraction mass and extraction efficiency. It is important to observe that different extraction processes produced singular botanical extracts, which means that each process was able to withdraw a specific quantity of secondary metabolites. Similarly to the stability problem previously described, in which 1 kg of commercial Neem oil may present different biological activities from another one in an advanced degradation stage, botanical extracts will certainly show a distinct action in insect control.

As a matter of fact, each botanical extract of Table 1 was assayed against *S.frugiperda* in concentrations of 100, 250 and 1000 mg of extract to 1,000 g of artificial diet (Forim et al., 2010b). The diet prepared at a concentration of 100 mg kg^{-1} by using the **MHE** extract shows the best results having 100% of mortality of *S.frugiperda* larvae. **EU** extract displayed a tendency to prolong the larval phase to 10 days, a reduction in 35% of the pupal weight, and presenting 70 % of mortality, approximately. The **EC** extract itself did not show biological activity. Results of this experiment are described in Table 4. In these experiments, it is easy to observe the importance of the composition of metabolites as azadirachtin. The higher the quantity of azadirachtin, the better were the results.

Samples prepared with 250 mg of extracts in 1,000 g of diet assayed against *S. frugiperda* also showed a relation between the content of azadirachtin and the insecticidal action. It is possible to observe in Figure 5 that the biological action increased along with the quantity of extracts in diets, *i.e.* the increase which occurred was in the content of azadirachtin, thus making the mortality of *S. frugiperda* larvae rise. Again, the **EC** extract showed the worst results against the target insect. Diets with 1,000 mg of extract in each 1,000 g (0,1% w/w)

showed 100% of mortality, except when prepared by using the **EC** extract (90 % of mortality after 15 days).

Extract	Larval phase (days)	Pupal phase (days)	Mean weight gained (mg)	Mortality (%)
EU	27.67	11.50	171.67	70.0
MV	17.80	11.00	251.60	60.0
MEA	19.12	10.62	217.12	20.0
ME	15.89	10.11	276.78	10.0
MM	16.10	10.00	267.90	10.0
Controle	17.56	9.56	267.89	10.0

Temperature: 25±2 °C; UR: 70 ± 5%; Fotophase of 12 h; After 10 days of incubation, mean of ten replicates (n = 10).

Table 4. Comparison of the efficacy of the diets prepared against larvae of *S. frugiperda* by using 100 mg kg^{-1} of different Neem extracts.

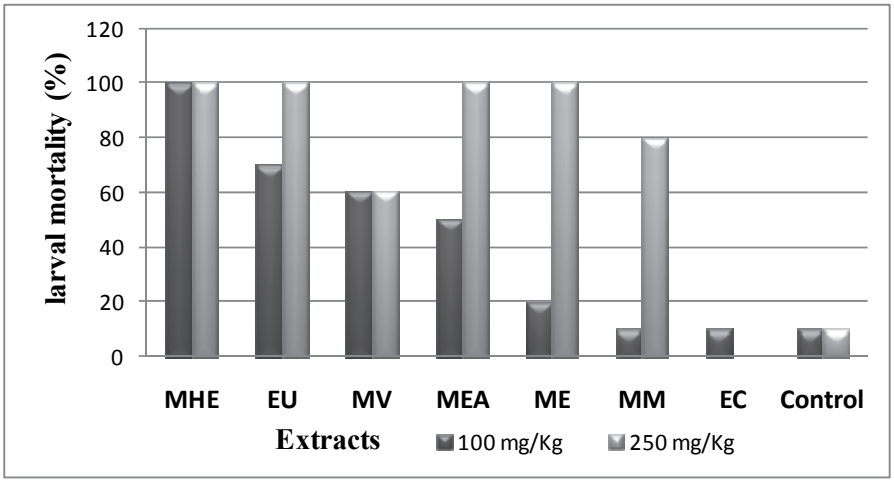


Fig. 5. Average mortality of *S. frugiperda* larvae fed by artificial diet prepared by using extracts of Neem at 100 and 250 mg kg^{-1}.

In these experiments, there is a strong relationship between the biological activity and the content of azadirachtin. Simple calculations of the amount of azadirachtin in diets showed that 100% of mortality was obtained just when this molecule was higher than 3 mg kg^{-1} (Table 5). These data establish a minimal limit of azadirachtin which needs to be applied to crops in order to reach the success on *S. frugiperda* control. Furthermore, these data may also be used in industrial.

Obviously, the relations above described could only be observed through the use of monitoring methods such as HPLC. At the same time, such relations reinforce the concern on natural products traditionally sold without any programs or processes of quality control.

Extract	Concentration of Azadiractin (mg kg^{-1})		
	100 mg kg^{-1} (Extract/Diet)	250 mg kg^{-1} (Extract/Diet)	1,000 mg kg^{-1} (Extract/Diet)
MHE	3,2	8,1	32,5
MM	1,2	3,0	12,1
ME	2,1	5,3	21,0
MEA	2,0	4,9	19,5
EU	2,9	7,4	29,5
EC	0,1	0,3	1,4
MV	1,8	4,6	18,5

Table 5. Concentration of azadirachtin in diets used in assays against *S. frugiperda*.

8. Formulations of botanical insecticides

Problems such as seasonal change, genetics, ontogenesis and production techniques may compromise the biological efficacy of natural products. Simple formulation methods and incorporation processes of botanical extracts into commercial products may minimize these problems. The commercial Neem oil is usually extracted from seed kernels of the Neem tree by pressing the seed kernels which are crushed and squeezed. This releases and separates the oil and the cake. Some of the active ingredients in Neem oil are susceptible to heat, therefore oil cold pressing is recommended. Completing the process, the remaining Neem seed cake is extracted with hexane. Obviously, for reasons already discussed before, the azadirachtin content in these commercial products may change constantly.

As it also occurs to synthetic pesticides, commercial products of botanical extracts need to be properly formulated. This development has led to a need for a wide range of product formulation types, additives and technological processes to prepare formulation of botanical extracts with various physical and chemical properties (Knowles, 2008).

Commercial Neem oil containing emulsifier agents may be formulated by using botanical extracts with a higher concentration of azadirachtin. Neem oils usually show a range of azadirachtin amounts from 300 to 1,500 mg kg^{-1}. In order for the product to be successful and viable to agricultural crops, it is necessary to always prepare the commercial formulations with the same content of this marker. Neem oils containing 1,000, 2,000 or 4,000 mg kg^{-1} of azadirachtin may be prepared by either the dilution process by using another poor Neem oil or Neem extracts containing a higher quantity of azadirachtin. For instance, specific quantities of Neem ethanolic extracts, easily prepared by phytochemical techniques with amounts of azadirachtin higher than 5%, may be incorporated in oil matrixes resulting in commercial products with reproducible contents of active compounds. Figure 6 shows chromatograms of enriched Neem oils which were prepared with different concentration of azadirachtin. In these formulations, specific quantities of extract and Neem oil were utilized, which had 61,698 mg kg^{-1} and 738 mg kg^{-1} of azadirachtin, respectively.

Again, these formulations and planning are only possible through the use of monitoring analytical methods, which also help in self-life studies. Otherwise, the commercial products would just be simple botanical extracts without any guarantee of biological activity reproducibility.

However, the ability of reproducing the content of a marker, many times, is not enough to guarantee the stability and quality of botanical extracts. By using the Neem as an example,

the azadirachtin is extremely labile in the presence of sunlight decreasing its biological and residual efficiency in field. In this case, knowing the chemical profile of Neem oil and/or extracts is not enough. For purposes of effective use, the azadirachtin molecule must be stabilized.

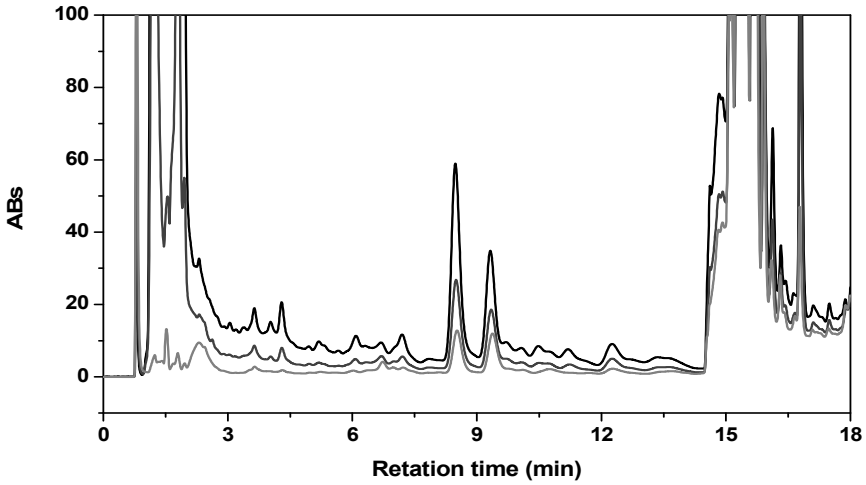


Fig. 6. Chromatograms of enriched Neem oil containing 1,000, 2,000 and 4,000 mg kg^{-1} of azadirachtin.

The addition of UV absorbing compounds to the formulations were found to protect photolabile pesticides, thereby extending their environmental life (Hussain, et al., 1990; Fowler et al., 1994). The UV absorbers can either absorb light preferably and prevent photo-excitation of the pesticide or accept the excess energy from the already excited pesticide molecules by different energy transfer or charge transfer mechanisms, thus extending the life-span of the molecule. Sundaram & Curry (1996) published a work by using UV absorbing compounds to stabilize the azadirachtin. Photostabilization of neem-based azadirachtin insecticide applied onto glass surfaces was studied in the presence of three UV absorbers, 2.4-dihydroxybenzophenone (Uvinul M-400, UM), 4-aminobenzoic acid (PABA), and fluorescent brightener-28 (FB-2X), a stilbene disulfonic acid derivative. The UV absorber UM, provided excellent protection, increasing the dissipation of half-life (DT_{50}) of pure azadirachtin from 3.87 to 22.54 days. On the other hand, the photostabilization due to PABA was marginal. The UV absorber, FB-2X acted as an effective photosensitizer, reducing the DT_{50} of azadirachtin from 3.87 to 0.31 days.

Kumar & Parmar (1999) formulated the azadirachtin employing either anthraquinone or epichlorohydrin as stabilizers in clay-based powders or Neem oil. Such compounds reduced the degradation rate by 26-60% compared to Neem oil, during the 14-day heat storage studies at 54 ± 1 °C in the laboratory. Other products used to control the azadirachtin stability were the addition of antioxidants such as ferulic acid, gallic acid, and rutin resulting in a moderate degree of photostabilization (Wei-Hong & Zhan-Qian, 2006). These examples show how the good laboratory practices may help during the development of commercial products of botanical extracts. They also highlight the importance of analytical methods to observe the associated phenomena.

The stability of Neem products have also been investigated through techniques of encapsulation in which several matrixes are used. Riyajan & Sakdapipanich (2009a) prepared capsules containing Neem extracts. Controlling the release of the biopesticide was achieved by the use of glutaraldehyde-alginate gel capsules modified by coating with a natural rubber layer. This work shows that the degree of release of azadirachtin (marker) from capsules into an aqueous environment was controlled by their formulation condition. In another work, Riyajan & Sakdapipanich (2009b) encapsulated Neem extracts into microcapsules by using hydrolyzed poly(vinyl acetate) crosslinked with glutaraldehyde by Spray-Drying technique to control its release and photodegradation stability. Sreenivasa et al. (2006) report an improved granular formulation of Neem seed extract having enhanced the storage stability, and the ability of a gradual release of azadirachtin for application to the plant rhizosphere. The formulations consist of an inert particle compound as a carrier, at least one lipophilic substance as a deactivator/binder, colorant and Neem extracts.

Nowadays, a recent technology has been revolutionizing the agribusiness: it is the nanotechnology. It has been presented in several areas of research such as material engineering (polymers, ceramic, metals), semiconductors, health and medicine, pharmaceutical, textiles, cosmetics, pesticides, *etc*. Among different techniques and concepts of nanoparticles, the polymeric nanoparticles have been a sophisticated approach towards agrochemical formulations, which may be applied into the search of new proprieties and efficient use of botanical extracts as Neem.

Nanoparticles are defined as colloidal polymeric particles containing an active compound including nanocapsules and nanospheres. Nanocapsules are carries composed of an oil core surrounded by a polymeric wall, whereas nanospheres consist of a polymeric matrix. Both colloids are stabilized by surfactants at the particle/water interface (Schaffazick et al., 2006). Both systems, nanocapsules and nanospheres, may be employed as support to delivery-controlled biopesticides programs or as stabilizer agents with several molecules such as azadirachtin. These systems can be prepared by methods based on the polymerization of dispersed monomers or the dispersion of a preformed polymer (Mora-Huertas et al., 2010).

In our laboratory, we have developed polymeric nanoparticles containing Neem oil and extracts. The main technique which has been employed is the modified interfacial deposition of a preformed polymer (nanoprecipitation) as described by Fessy et al. (1989). This method is based on the interfacial deposition of a polymer following the displacement of a semi-polar solvent miscible with water from a lipophilic solution. In these works, nanoparticles, nanocapsules and nanospheres, in colloidal suspension and in powders have been produced. The powders have been produced through Spray-drying techniques and by using drying inorganic support. The aims have been to enhance storage and UV stability for azadirachtin, to improve its dispersion in aqueous phases and to control the ability of azadirachtin release. The polymers employed are usually biopolymers, *i.e.* polymers which are biodegradable in a brief time. Whenever, a botanical extract is formulated, it is very important to employ materials which are compatible with desired features of natural products such as being environment-friendly. Figure 7 shows nanoparticles of Neem prepared by this technique.

Through the use of different quantities of Neem extracts in nanoparticles formulations, which have higher azadirachtin concentration, it was possible to prepare colloidal suspensions containing a larger range of this marker. The values of absolute recovery and entrapment efficiency for three nanoparticles formulations containing different azadirachtin concentrations are described in Table 6. Nanoencapsulated agrochemicals should be

designed in such a way that they possess all necessary properties such as effective concentration (with high solubility, stability and effectiveness), time controlled release in response to certain stimuli, enhance targeted activity and less ecotocity with safe and easy mode of delivery thus avoiding repeated application (Nair et al., 2010).

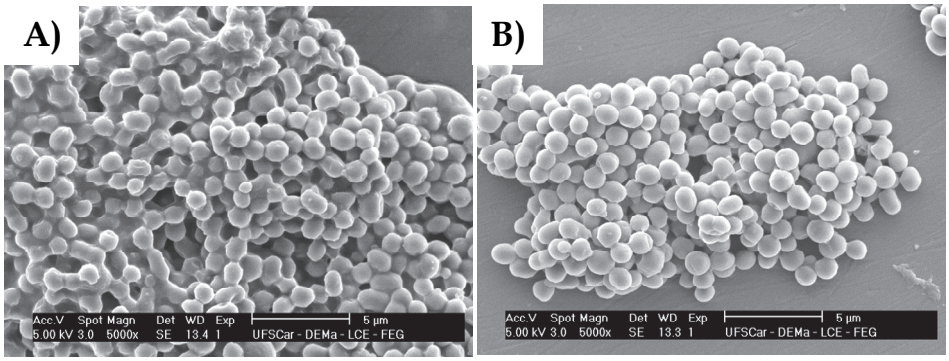


Fig. 7. Photomicroscopy of A) nanocapsules and B) nanospheres of PCL loaded with Neem extracts.

Formulation	Nominal concentration (µg ml^{-1})	Absolute recovery (%)	Entrapment efficiency (%)
01	2.200,0	102,2 ± 1,89	98,7 ± 0,01
02	2.800,0	99,2 ± 1,03	98,8 ± 0,04
03	3.400,0	95,8 ± 2,00	98,8 ± 0,01

The values were expressed as average result ± standard deviation (n = 3)

Table 6. Quantitative analysis of nanocapsules containing Neem extracts in colloidal suspension.

It is possible to observe that the nanoparticle production process did not affect the azadirachtin stability. Another important information in Table 4 is that the quantity of azadirachtin in formulation number 3 into a colloidal aqueous phase represents thirteen times the capacity of the azadirachtin to be solubilized in water. When nanocapsules and nanospheres were prepared, the average particle sizes were nearly 240 nm and 120 nm, respectively.

Through the Spray-drying technique nanoparticles containing Neem extract in powders were obtained. This process removes the water presence thus increasing the stability of the Neem product. Colloidal suspension and dried material were subjected to UV stability assays. This experiment was carried out in a mirror camera at 30 °C. The results are illustrated in Figure 8. This experiment shows that azadirachtin nanoencapsulated was more stable, confirming the importance of the use of an appropriate formulation. Furthermore, the products without water show a greater stability.

Obviously, more stable products will present a larger time of self-life and a higher residual action in fields. They will have ways to conserve the chemical profile and active molecules of botanical compounds causing direct impacts on the biological action and their efficacy in

field against insect pests. Thus, enriched or micro/nano-structured natural products are important tools to control the botanical action of these products in field.

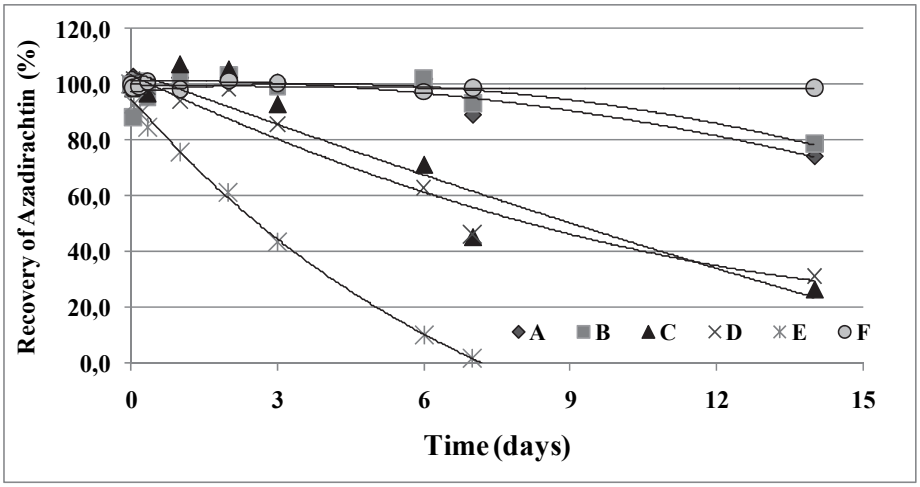


Fig. 8. Recovery of azadirachtin after UV radiation. A) Nanocapsules in powder without tensoactive; B) Nanocapsules in powder with tensoactive; C) Nanocapsules in colloidal suspension without tensoactive; D) Nanocapsules in colloidal suspension with tensoactive; E) Neem oil and F) Neem oil coated of UV radiation.

These results intensify the idea of the importance of correct formulations which need to be applied to obtain commercial botanicals. Moreover, these results also show the importance of the use of analytical methods during the development of botanical products. Through analytical methods, it is possible to monitor all the steps of development

9. Conclusions

Sustainable growth in agriculture is crucial for most of the developing countries to provide for the growing populations. Synthetic chemicals for crops protection are associated with pest resurgence, impact on non-target organisms, health and environment. Hence there is the need to develop safe alternative crop protectants, which should be more specific and cover a larger range of activities (Nathan et al., 2006c). A large number of different plant species representing different geographical areas around the world have shown to possess phytochemicals (secondary metabolites) that are capable of causing a range of insecticidal effects. Through the use of these plants it is possible to obtain botanical extracts which may be utilized in commercial bioinsecticidal formulations. Among these species, the *Azadirachta indica* has had an outstanding position. However, problems such as seasonal changes, genetics, ontogenesis and production techniques may compromise the reproducibility and biological efficacy of botanical extracts. These limitations have been easily observed through analysis in different Neem products. Such results enhance the importance of studies about preparation, manipulation, storage, formulation, and quality control of natural products before their agricultural application.

These problems may be minimized by using appropriate formulations. It is possible to use the techniques of enrichment, application of additives, or developing micro or nano formulations. Furthermore, real control in commercial natural products is just possible by using monitoring analytical methods. Chromatography methods of analysis have the potential to determine the quali and quantitative chemical profile. The method used is required to identify the active or marker compounds, composition analysis and fingerprinting purposes. The complex relation between the chromatographic analysis and efficacy of botanical extracts is the most important aspect for the quality control of botanical products. Chemical analysis of extracts from plant material will play a central role in the development and modernization of biopesticides. Chemical fingerprints or content of markers analysis might be linked to biological assays to provide assurance of efficacy and consistency of botanical extracts (Liang et al., 2004).
Thus, the researches concerning the relation between the chromatographic analysis and formulated extracts to efficacy of bioinsecticides are urgent requirements for the quality control of botanical products.

10. Acknowledgments

The authors are grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), for the financial support.

11. References

Alkofani, A., Rupprecht, J.K., Anderson, J.E., McLaughlin, J.L., Mikolajezak, K.L., & Scott, B.A. (1989). Search for New perticides from Higher Plants. In.: *Insecticides of Plant Origin*, Arnason, J.T., Philogène, B.J.R., Morand, P. pp. 25-43, ACS Symposium Sereis 387, ISBN 9780841215696, Washington.

Arimura, G., Kost, C., & Boland, W. (2005). Herbivore-induced, indirect plant defences. *Biochimica & Biophysica Acta,* 1734, pp. 91-111.

Barrek, S. Paisse, O., & Grenier-Loustalot, M-F. (2004) Analysis of Neem Oils by LC-MS and Degradation Kinetics of Azadirachtin-A in a Controlled Environment. Characterization of Degradation Products by HPLC-MS-MS. *Anul Bioanal. Chem*., 378, pp. 753-763.

Benerji, B. & Nigam, S.K. (1984).Wood constituents of Meliaceae: a review. *Fitoterapia,* 55, pp. 3-36.

Butterworth, J.H., &; Morgan, E.D. (1971). Investigation of the locust feeding Inhibition of the seeds of the Neem Tree, Azadirachta indica. *J. Insect Physiol*., 1971, pp. 969-977.

Carson, R.L. (1962). *Silent Spring*. Riverside Press, Cambridge.

Céspedes, C.L., Torres, P., Marín, R.C., Arciniegas, A., Vivar, A.M., Pérez-Castorena, A.L., & Aranda, E. (2004). Insect growth inhibition by tocotrienols and hydroquinones from Roldana barba-johannis. *Phytochemistry,* 65, pp. 1963–1975.

Céspedes, C.L., Salazar, J.R., Martínez, M., & Aranda, E. (2005). Insect growth regulatory effects of some extracts and sterols from *Myrtillocactus geometrizans* (Cactaceae) against *Spodoptera frugiperda* and *Tenebrio molitor*. *Phytochemistry,* 66, pp.2 481–2493.

Charleston, D.S., Kfir, R., Dicke, M., & Vet, L.E.M. (2006). Impact of botanical extracts derived from *Melia azedarach* and *Azadirachta indica* on populations of *Plutella*

xylostella and its natural enemies: A Weld test of laboratory findings. *Biological Control,* 39, pp. 105–114.

Dayan, F.E., Cantrell, C.L., & Duke, S.O. (2009). Natural Products in Crop Protection. *Bioorganic & Medicinal Chemistry,* 17, pp. 4022–4034.

Dewick, P.M. (2009). *Medicinal Natural Products: A Biosynthetic Approach*. 3ª Ed. John Wiley & Sons, ISBN: 9780470741689, Chichester.

Dixon, R.A., & Strack, D. (2003). Phytochemistry meets genome analysis, and beyond. *Phytochemistry*, 62, pp. 815-816.

Du, Y-D., Jia, R-Y., Yin, Z-Q., Pu, Z-H., Chen, J., Yang, F., Zhang, Y-Q., & Lu, Y. (2008). Acaricidal activity of extracts of neem (*Azadirachta indica*) oil against the larvae of the rabbit mite *Sarcoptes scabiei* var. cuniculi in vitro. *Veterinary Parasitology,* 157, pp. 144–148.

Duke, S.O., Romagni, J.G., & Dayan, F.E. (2000). Natural products as sources for new mechanisms of herbicidal action. *Crop Protection,* 19, pp. 583-589.

Feng, R., & Isman, M.B. (1995). Selection for resistance to azadirachtin in the green peach aphid Myzus persicae. *Experientia*, 51, pp. 831-833.

Fessi, H., Puisieux, F., Devissaguet, J-P. Ammoury, N., & Benita, S. (1989). Nanocapsule formation by interfacial deposition following solvent displacement. *Int. J. Pharm.* 55, pp. R1- R4.

Forim, R.F., da Silva, M.F.G.F., Cass, Q.B., Fernandes, J.B., & Vieira, P.C. (2010a). Simultaneous quantification of azadirachtin and 3-tigloylazadirachtol in Brazilian seeds and oil of Azadirachta indica: application to quality control and marketing. *Analytical Methods,* 2, pp. 860–869.

Forim, M.R., Matos, A.P., da Silva, M.F.G.F., Cass, Q.B., Vieira, P.C., & Fernandes, J.B. (2010b). Uso de CLAE no controle de qualidade em produtos comerciais de nim: reprodutibilidade da ação inseticida. *Quimica Nova*, 33, 5, pp. 1082-1087.

Fowler, J.D., Feinstein, B.E., & Rolland, L.L. (1994). VIIl IUPAC Internat. Congr. Pesticide Chem., Washington, D.C., Vol. I, Abstr. No. 388, p. 437.

Gay, D.E., Pallardy, S.G., Garret, H.E., & Rottinghaus, G.E. (2003). Acute drought stress and plant age effects on alkamide and phenolic acid content in purple coneflower roots. *Plant Med*., 69, pp. 1024-1030.

Gobbo-Neto, L., & Lopes, N.P. (2007). Plantas medicinais: fatores de influência no conteúdo de metabólitos secundários. *Química Nova,* 30, pp. 374-381.

Gopalakrishnan, G., Singh, N.D.P., & Kasinath, V. (2001). Photomediated Transformation of Salannin, a tetranortriterpenoid from *Azadirachta indica* A. Juss. *Molecules*, 6, pp. 551-556.

Harvey, A.L. (2007). Natural products as a screening resource. *Current Opinion in Chemical Biology*, 11, pp. 480–484.

Hayves, K.F. (1988). Sublethal effects of neurotoxic insecticides on insect behaviour. *Annual Review of Entomology,* 33, pp. 149-168.

Hussain, M., Perschke, H., & Kutscher, R., (1990). The Effect of Selected UV Absorber Compounds on the Photodegradation of Pyrethroid Insecticides Applied to Cotton Fabric Screens. *J. Pesticide Sci*., 28, pp. 345-355.

ICH [International Conference on Harmonization, FDA, USA]. (1996). Tittle, In: *Q2B: Validation of Analytical Procedures: Methodology.* Jul/02/2011. Available from:

<http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM073384.pdf)>.

Isman, M.B. (1997). Neem and other botanical insecticides: barriers to commercialization. *Phytoparasitica,* 25, pp. 339-344.

Jadeja, G.C., Maheshwari, R.C., & Naik, S.N. (2011). Extraction of natural insecticide azadirachtin from neem (*Azadirachta indica* A. Juss) seed kernels using pressurized hot solvent. *J. of Supercritical Fluids,* 56, pp. 253–258.

Javed, N., Gowen, S.R., El-Hassan, S.A., Inam-ul-Haq, M., Shahina, F., & Pembroke, B. (2008). Efficacy of neem (*Azadirachta indica*) formulations on biology of root-knot nematodes (*Meloidogyne javanica*) on tomato. *Crop Protection,* 27, pp. 36–43.

Kéita, S.M., Vincent, C., Schmit, J-P., Arnason, J.T., & Bélanger, A. (2001). Efficacy of essential oil of *Ocimum basilicum* L. and *O. gratissimum* L. applied as an insecticidal fumigant and powder to control *Callosobruchus maculatus* (Fab.) [Coleoptera: Bruchidae]. *Journal of Stored Products Research,* 37, pp. 339–349.

Knowles, A. (2008). Recent developments of safer formulations of agrochemicals. *Environmentalist,* 28, pp. 35-44.

Kumar, Ch.S.S.R., Srinivas, M., & Yakkundi, S. (1996). Limonoids from the Seeds of *Azadirachta indica*. *Phytochemistry*, 43, pp. 451-455.

Kumar, J., & Parmar, B.S. (1999). Stabilization of Azadirachtin A in Neem Formulations: Effect of Some Solid Carriers, Neem Oil, and Stabilizers. *J. Agric. Food Chem*., 47, pp. 1735-1739.

Kumar, A.R.V., Jayadevi, H.C., Ashoka, H.J., & Chandrashekara, K. (2003). Azadirachtin use efficiency in commercial neem formulations. *Current science,* 84, pp. 1459-1464.

Lale, N.E.S., & Abdulrahman, H.T. (1999). Evaluation of neem (*Azadirachta indica* A. Juss) seed oil obtained by diferent methods and neem powder for the management of *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) in stored cowpea. *Journal of Stored Products Research,* 35, pp. 135-143.

Lee, S.M., Olsen, J.I., Schweizer, M.P., & Klocke, J.A. (1988). 7-Deacetyl-17β-hydroxyazadiradione, a new limonoid insect Growth inhibitor from *Azadirachta indica*. *Phytochemistry*, 21, pp. 2113-2115.

Liang, Y-Z., Xie, P., & Chan, K. (2004). Quality control of herbal medicines. *Journal of Chromatography B* 812, pp. 53-70.

Liu, Z.L., & Ho, S.H. (1999). Bioactivity of the essential oil extracted from *Evodia rutaecarpa* Hook f. et Thomas against the grain storage insects, *Sitophilus zeamais* Motsch. and *Tribolium castaneum* (Herbst). *Journal of Stored Products Research,* 35, pp. 317-328.

Lobstein, A., Jako, L.R., Berrurier, M.H., & Anton, R. (1991). Seasonal Variations of the Flavonoid Content from *Ginkgo biloba* Leaves. *Planta Med*. 57, pp. 430-433.

Luo, X., Ma, Y., Wu, S., & Wu, D. (1999). Two novel azadirachtin derivatives from *Azadirachta indica*. *J. Nat. Prod*., 62, pp. 1022-1024.

Mancebo, F., Hilje, L., Mora, G.A., & Salazar, R. (2002). Biological activity of two neem (*Azadirachta indica* A. Jus., Meliaceae) products on *Hypsipyla grandella* (Lepidoptera: Pyralidae) larvae. *Crop Protection,* 21, pp. 107–112.

Mann, J. (2005). *Secondary Metabolism*. 2ª Ed.: Oxfort University Press, ISBN 0198555296, New York.

Montes-Molina, J.A., Luna-Guido, M.L., Espinoza-Paz, N., Govaerts, B., Gutierrez-Miceli, F.A., & Dendooven, L. (2008). Are extracts of neem (*Azadirachta indica* A. Juss. (L.))

and *Gliricidia sepium* (Jacquin) an alternative to control pests on maize (*Zea mays* L.)? *Crop Protection,* 27, pp. 763-774.

Mora-Huertas, C.E., Fessi, H., & Elaissari, A. (2010). Polymer-based nanocapsules for drug delivery. *International Journal of Pharmaceutics,* 385, pp. 113-142.

Morgan, E.D. (2009). Azadirachtin, a scientific gold mine. *Bioorganic & Medicinal Chemistry,* 17, pp.4096–4105.

Nair, R., Varghese, S.H., Nair, B.G., Maekawa, T., Yoshida, Y., & Kumar, D.S. (2010). Nanoparticles material delivery to plants. *Plant Science,* 179, pp. 154-163.

Nathan, S.S., Kalaivani, K., & Sehoon, K. (2006a). Effects of *Dysoxylum malabaricum* Bedd. (Meliaceae) extract on the malarial vector *Anopheles stephensi* Liston (Diptera: Culicidae). *Bioresource Technology,* 97, pp. 2077–2083.

Nathan, S.S., & Sehoon, K. (2006b). Effects of *Melia azedarach* L. extract on the teak defoliator *Hyblaea puera* Cramer (Lepidoptera: Hyblaeidae). *Crop Protection,* 25, pp. 287–291.

Nathan, S.S., Kalaivani, K., & Murugan, K. (2006c). Effect of biopesticides on the lactate dehydrogenase (LDH) of the rice leaffolder, *Cnaphalocrocis medinalis* (Guenée) (Insecta: Lepidoptera: Pyralidae). *Ecotoxicology and Environmental Safety,* 65, pp. 102–107.

Nathan, S.S., Choi, M.Y., Paik, C.H., Seo, H.Y., Kim, J.D., & Kang, S.M. (2007). The toxic effects of neem extract and azadirachtin on the brown planthopper, *Nilaparvata lugens* (Stal) (BPH) (Homoptera: Delphacidae). *Chemosphere,* 67, pp. 80–88.

Negahban, M., Moharramipour, S., & Sefidkon, F. (2006). Chemical Composition and Insecticidal Activity of *Artemisia scoperte* Essential Oil against Three Coleopteran Stored-Product Insects. *J. Asia-Pacific Entomol*., 9, pp. 381-388.

Nugroho, B.W., Edrada, R.A., Wray, V., Witte, L., Bringmann, G., Gehling, M., & Proksch. P. (1999). An insecticidal rocaglamide derivatives and related compounds from *Aglaia odorata* (Meliaceae). *Phytochemistry,* 51, pp. 367-376.

Ogendo, J.O., Kostyukovsky, M., Ravid, U., Matasyoh, J.C., Deng, A.L., Omolo, E.O., Kariuki, S.T., & Shaaya, E. (2008). Bioactivity of *Ocimum gratissimum* L. oil and two of its constituents against five insect pests attacking stored food products. *Journal of Stored Products Research,* 44, pp. 328–334.

Ong, E.S. (2004). Extraction methods and chemical standardization of botanical and herbal preparations. *Journal of Chromatography B*, 812, pp. 23-33.

Panagiotakopulu, E., Buckland, P.C., & Day, P.M. (1995). Natural Insecticides and Insect Repellents in Antiquity: A Review of the Evidence. *Journal of Archaeological Science*, 22, pp. 705-710.

Rattan, R.S. (2010). Mechanism of action of insecticidal secondary metabolites of plant origin. *Crop protection,* 29, pp. 913-920.

Rharrabe, K., Amri, H., Bouayad, N., & Sayah, F. (2008). Effects of azadirachtin on post-embryonic development, energy reserves and ∝-amylase activity of *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae). *Journal of Stored Products Research,* 44, pp. 290– 294.

Rijke, E., Out, P., Niessen, W.M.A., Ariese, F., Gooijer, C., & Brinkman, U.A.Th. (2006). Analytical separation and detection methods for flavonoids. *Journal of Chromatography A*, 1112, pp. 31-63.

Riyajan, As-Ad., &; Sakdapipanich, J.T. (2009a). Developmento of a controlled release neem capsule with a sodium alginate matrix, crosslinked by glutaraldehyde and coated with natural rubber. *Polym. Bull*., 63, pp. 609-622.

Riyajan, As-Ad. & Sakdapipanich, J.T. (2009b). Encapsulated neem extract containing Azadiractin-A within hydrolyzed poly(vinyl acetate) for controlling its release and photodegradation stability. *Chemical Engineering Journal,* 152, pp. 591-597.

Roark, RC. (1947). Some promising insecticidal plants. *Econ. Bot.* 1, pp. 437-445; *apud* Shaalan, E.A-S., Canyon, D., Younes, M.W.F., Abdel-Wahab, H., & Mansour, A-H. A review of botanical phytochemicals with mosquitocidal potential. *Environment International,* 31, pp. 1149 – 1166.

Roel, A.R., Dourado, D.M., Matias, R., Porto, K.R.A., Bednaski, A.V., & da Costa, R.B. (2010). The effect of sub-lethal doses of *Azadirachta indica* (Meliaceae) oil on the midgut of *Spodoptera frugiperda* (Lepidoptera, Noctuidae). *Revista Brasileira de Entomologia*, 54, pp. 505–510.

Ruiu, L., Satta, A., & Floris, I. (2008). Effects of an azadirachtin-based formulation on the non-target muscoid fly parasitoid *Muscidifurax raptor* (Hymenoptera: Pteromalidae). *Biological Control,* 47, pp. 66–70.

Saxena, R.C. (1989). Insecticides do neem. In: *Insecticides of Plant Origin,* Arnason, J.T., Philogène, B.J.R., & Morand, P., pp. 110-135, ACS Symposium Series, 387, ISBN 9780841215696, Washington.

Schaffazick, S.R., Pohlmann, A.R., Mezzalira, G., & Guterres, S.S. (2006). Development of nanocapsule suspensions and nanocapsule spray-dried powders containing melatonin. *J. Braz. Chem. Soc*., 17, pp. 562-569.

Schmidt, B., Ribnicky, D.M., Poulev, A., Logendra, S., Cefalu, W.T., & Raskin, I. (2008). A natural history of botanical therapeutics. *Metabolism Clinical and Experimental,* 57, (Suppl 1), pp. S3–S9.

Schmutterer, H. (1988). Potential of azadirachtin-containing pesticides for integrated pest control in developing and industrialized countries. *J. Insect Physiol*., 34, pp. 713-719.

Seljasen, R., & Meadow, R. (2006). Effects of neem on oviposition and egg and larval development of *Mamestra brassicae* L: Dose response, residual activity, repellent effect and systemic activity in cabbage plants. *Crop Protection,* 25, pp.338–345.

Shaalan, E.A-S., Canyon, D., Younes, M.W.F., Abdel-Wahab, H., & Mansour, A-H. (2005). A review of botanical phytochemicals with mosquitocidal potential. *Environment International*, 31, pp. 1149–1166.

Sharma, V., Walia, S., Kumar, J., Nair, M.G., & Parmar, B.S. (2003). An efficient method for the purification and characterization of nematicidal azadirachtins A, B, and H, using MPLC and ESIMS. *J. Agric. Food Chem*., 51, pp. 3966-3972.

Shidu, O.P., Kumar, V., & Behl, H.M. (2003). Variablity in Neem (*Azadirachta indica*) with Respect to Azadirachtin Content. *J. Agric. Food Chem*., 51, pp. 910-915.

Siddiqui, B.S., Ghiasiddin., Faizi, S., & Rasheed, M. (1999). Triterpenoids of fruit coats of *Azadirachta indica. J. nat. Prod*., 62, pp. 1006-1009.

Siddiqui, B.S. Afshan, F., & Faizi, S. (2001). Three novel tetracyclic triterpenoids of biogenetic interest from the leaves of *Azadirachta indica. Tetrahedron*, 57, pp. 10281-10286.

Sieber, K.-P., & Rembol, H. (1983). The effects of azadirachtin on the endocrine control of moulting in *Locusta migratoria. J. lnsrcr Physiol*., 29, pp. 523.-527. 1983

Southwell, I.A. & Bourke, C.A. (2001). Seasonal variation in hypericin content of *Hypericum perforatum* L. (St John's wort). *Phytochemistry*, 56, pp. 437-441.

Sreenivasa, R.D., Davendra, K., & Liza, J., Chandrasekaran, G.M. (2006). Granular formulation of neem seed extract and its process thereof. United States Patent 20060099233.

Strege, M.A. (1999). High-performance liquid chromatography-electrospray ionization mass spectrometric analysis for the integration of natural products with modern high-throughput screening. *Journal of Chromatography B,* 725, pp. 67-78.

Sundaram, K.M.S. & Curry, J. (1996). Effect of Some Uv Light Absorbers On The Photostabilization Of Azadirachtin, A Neem-Based Biopesticide. *Chemosphere*, 32, pp. 649-659.

Sundaram, K.M.S., & Curry, J. (1994). Initial deposits and persistence of azadirachtin in fir and oak foliage. *J. Pestic. Sci*., 41, pp. 129-138.

Thacker, J.R.M., Bryan, W.J., McGinley, C., Heritage, S., Strang, R.H.C. (2003). Field and laboratory studies on the effects of neem (*Azadirachta indica*) oil on the feeding activity of the large pine weevil (*Hylobius abietis* L.) and implications for pest control in commercial conifer plantations. *Crop Protection,* 22, pp. 753–760.

Thejavathi, R., Yakkundi, S.R., & Ravindranath, B. (1995). Determination of Azadirachtin by Reversed-phase High-Performance Liquid Chromatography Using Anisole as Internal Standard. *Journal Chromatography A*., 705, pp. 374-379.

Torres, P., Avila, J.G., de Vivar, A.R., García, A.M., Marín, R.C., Aranda, E., & Céspedes, C.L. (2003). Antioxidant and insect growth regulatory activities of stilbenes and extracts from *Yucca ericulosa. Phytochemistry,* 64, pp. 463–473.

van der Werf, H.M.G. (1996). Assessing the impact of pesticides on the environment. *Agriculture, Ecosystems and Environment,* 60, pp. 81–96.

Viana, P.A, & Prates, H.T. (2003) Desenvolvimento e mortalidade larval de *Spodoptera frugiperda* em folhas de milho Tratadas com extrato aquoso de folhas de *Azadirachta indica. Bragantia,* 62, pp. 69-74.

Wallart, T.E., Pras, N., Beekman, A.C., Quax, W.J. (2000). Seasonal variation of artemisinin and its biossynthetic precursors in plants of Artemisia annua of different geographical origin: proof for the existance of chemotypes. *Planta Med*., 66, pp. 57-62.

Wei-Hong, T., & Zhan-qian, S. (2006). Research on effect of four natural ultraviolet light absorbers on photostabilization of azadirachtin-A. *Agric. Sci. China,* 5, pp. 855-858.

Yang, R. Z., & Tang, C.-S. (1988). Plants used for pest control in China: a literature review. *Econ. Bot*., 42, pp. 376-406.

Zarate, R., El Jaber-Vazdekis, N., Cequier-Sanchez, E., Gutierrez-Nicolás, F., & Ravelo, A.G. (2003). Biotechnology for the production of plant natural products. *Studies in Natural Products Chemistry*, 34, pp. 308-392.

Wei-Hong, T., & Zhan-Qian, S. (2006). Research on effect of four natural ultraviolet light absorbers on pphotostabilization of azadirachtin-A. *Agric. Sci. China,* 5, pp. 885-894.

17

Comparative Results of Action of Natural and Synthetic Acaricides in Reproductive and Salivar Systems of *Rhipicephalus sanguineus* - Searching by a Sustainable Ticks Control

Maria Izabel Camargo Mathias
UNESP,
Rio Claro, SP,
Brasil

1. Introduction

Ticks have been well studied and are one of the most important groups amongst the Arthropod, showing that there is of great interest in the medical and veterinarian aspects, mainly because of the fact that they appear to be carriers of many illnesses that attack domestic and wild animals, as well as humans (Sonenshine, 1991).
The ticks in rural or urban environment represent a big problem for society. For dairy producers and especially for meat and leather producer these ectoparasites cause huge financial losses due to their fixation in its hosts. Already in urban environments infestations in domestic dogs, as well as common contaminate the home environment, through the transmission of serious diseases to man.

2. *Rhipicephalus sanguineus* ticks

The importance of the ticks meanly *Rhipicephalus sanguineus* in the domestic world can be explained by the introduction of dogs in houses, for example, as companion animals consequently its ectoparasites facilitates the propagation of biopathogens those cause diseases both to the dogs and the human being; therefore these ticks are nowadays considered "urban plagues"
Numerous studies are currently under way to find an effective control strategy that would minimize the damage caused by these ectoparasites. A new tick control approach is an immunological one consisting in the identification, isolation and synthesis of proteins those protect tissues and organs of the tick, mainly those of the reproductive and glandular systems, with the aim of developing a vaccine (Tellam et al, 1992; Willadsen, 1997). However, nowadays the most efficient method to control tick populations is by suing chemical compounds, such as permethrin, fipronil and selamectin (active ingredients of different acaricides) which frequently act in the nervous system of the ticks (Mencke et al., 2003).

3. Ticks control

The challenge of controlling these arthropods has long been subject of several studies and the results obtained have been the formulation of new acaricides (synthetic chemicals) that are now widely used. However, collateral damages has been reported, including the emergence of individuals resistant to the drugs applied, which raises the source of new generations of ticks those are little affected by application of the acaricides. Also in this demand, the pharmaceutical industry (meanly of veterinary products) frequently releasing acaricides in an attempt to circumvent this problem. However, specific studies on the tick's cell biology are still scarce and little is known about the consequences of its utilization in non-target organisms.

3.1 Permethrin acaricide

The toxicity of an acaricide is defined as extent or degree to which a chemical substance to kill or injure the target organism. In this way, the toxicity of a drug is determined by running laboratorial tests on animals and it is expressed as LD_{50} (lethal dose fifty) and LC_{50} (lethal concentration fifty) values and are the amount or concentration, respectively, of the pesticide's active ingredient that is required to kill 50% of the tested animals under standardized tests conditions (Garcia-Garcia et al., 2005). The first study to described a detailed protocol of laboratorial procedures to determinate de LC_{50} of permethrin using semi-engorged females of *R. sanguineus* ticks was performed by Roma et al. (2010) who established the LC_{50}=2062 ppm (1.549-2.675). Acaricides currently available in the market have permethrin (chemical substance that cause a nervous impulses disorders and the ticks suffer excitement, indicated by tremors and spasms followed by paralysis and death) as active ingredient in concentration higher than 300.000 ppm and according Roma et al. (2010) permethrin in lower concentrations (approximately 100 times less) would be enough to kill *R. sanguineus* ticks, although this process would be slower.

3.2 Permethrin x *Rhipicephalus sanguineus* salivary gland

The feeding success of the ticks is the result of the action of the metabolism of the salivary glands, organs that are responsible for the fixation of the tick in the host (Moorhouse and Tatchell, 1966), by osmoregulation (Sonenshine, 1991), the inhibition of the host defense mechanisms such as coagulation and inflammation (Nuttal and Strickland, 1908: Paesen et al., 1999) and by the digestion of the tissues (Walker et al., 1985). Therefore, salivary glands are organs indispensable for the feeding process and therefore make ticks very biological successful organisms.

The tick's salivary glands are paired organs found in its celomatic cavity and contain approximately 1400 acini each (Walker et al., 1985). Many histological descriptions of salivary glands have been made in different species of ixodides ticks including by Furquim et al (2007, 2008). In summary, there are three different types of acini in females (I, II, III) and four in males (I, II, III and IV) and type I acini cells are agranular and have the osmoregulatory process as the main function, while types II, III and IV acini cells are granular and responsible for the other previously mentioned functions.

According to Nodari et al. (2011) the permethrin chemical induced morphophysiological changes in salivary glands of *R. sanguineus* semi-engorged females, even at lower concentrations, causing tissue changes compromising the organ metabolism, essential to the

feed process of the tick. The salivary glands in individuals subjected to 206 ppm of permetrhrin presented acini I morphologically altered (irregular shape and dilated lumen) corroborating Pereira et al. (2011), who reported that these acini in the same tick species were also affected by fipronil acaricide. These authors suggested that the acini I would be ormoregulators and through the saliva, they could remove the toxic compound from the hemolimph, since the lumen diameter of the acini suffer a significant increase. This authors showed that females subjected to permethrin at 2062 ppm presented only few acini I, with the other ones being classified as indeterminate, since they have lost their morphological and histological characteristics due the degeneration process by the toxic agent. The Nodari et al. (2011) results confirmed that permethrin, besides the proven neurotoxic action, also accelerates glandular tissue degeneration, an event that will occur naturally and with greater intensity only after full female engorgement (Fig. 1,2).

3.3 Fipronil x *Rhipicephalus sanguineus* salivary glands

In this sense, Oliveira et al. (2011) performed another study bringing information about the action of fipronil acaricide, using *R. sanguineus* as a biological model. Fipronil is a broad-spectrum chemical agent available in the market in a range of formulations from attractive baits for ant and cockroach control to sprays used in veterinary products for the control of ticks, fleas and mites (Penaliggon, 1997; Hugnef et al,. 1999; Higgins et al. 2001) and his effect are not fully known, as well as, the direct and indirect consequences of its use. The fipronil act mainly in the ticks GABA system, consisting of inhibitory neurotransmitters of neuromuscular junctions and central nervous system synapses (Denny, 2001). The authors developing an appropriate protocol for an in vitro bioassay (AIT) monitored daily. The LC_{50} and the 95% confidence interval were also determined. The increase in the concentration of fipronil (1ppm- 100 ppm) caused a progressive increase in the mortality of semi-engorged *R. sanguineus* females.

Pereira et al. (2011) in a study performed with the same chemical substance showed the action of fipronil in salivary glands of the same species females subjected to different concentrations. The acaricide accelerated the natural degeneration process of these glands, as well as reduced the number of the acini present in the gland as the concentration of the chemical compound is increased, making the organ unable to secrete its final saliva. From this process only the glandular ducts are preserved probably for being resistant to fipronil and having all the ducts (acinar, intermediate and common excretory ones) with lumen covered by cuticle, which prevented both the diffusion and the action of the acaricide on the epithelial cells. In the acini with intact cells the organelles were intact too (Fig. 3). In the altered cells there was evidence of apoptotic cell death which characteristic is to affect the cells individually and as synchronically. One common morphological characteristic of the cytoplasm of salivary gland of the *R. sanguineus* is the spherocrystal presence, constituted by phosphate and calcium carbonate and originated from the cisternae of the rough endoplasmic reticulum, however despite their presence in various systems of different species, no author stated what would be their real function (reserve of calcium, detoxication, ionic balance, etc.). In females subjected to 10 ppm of fipronil the glandular damages were extensive, considering that even the most resistant structure- the spherocrystals- became disorganized, resulting in a structure that went from perfectly round to completely irregular (Fig. 4, 5).

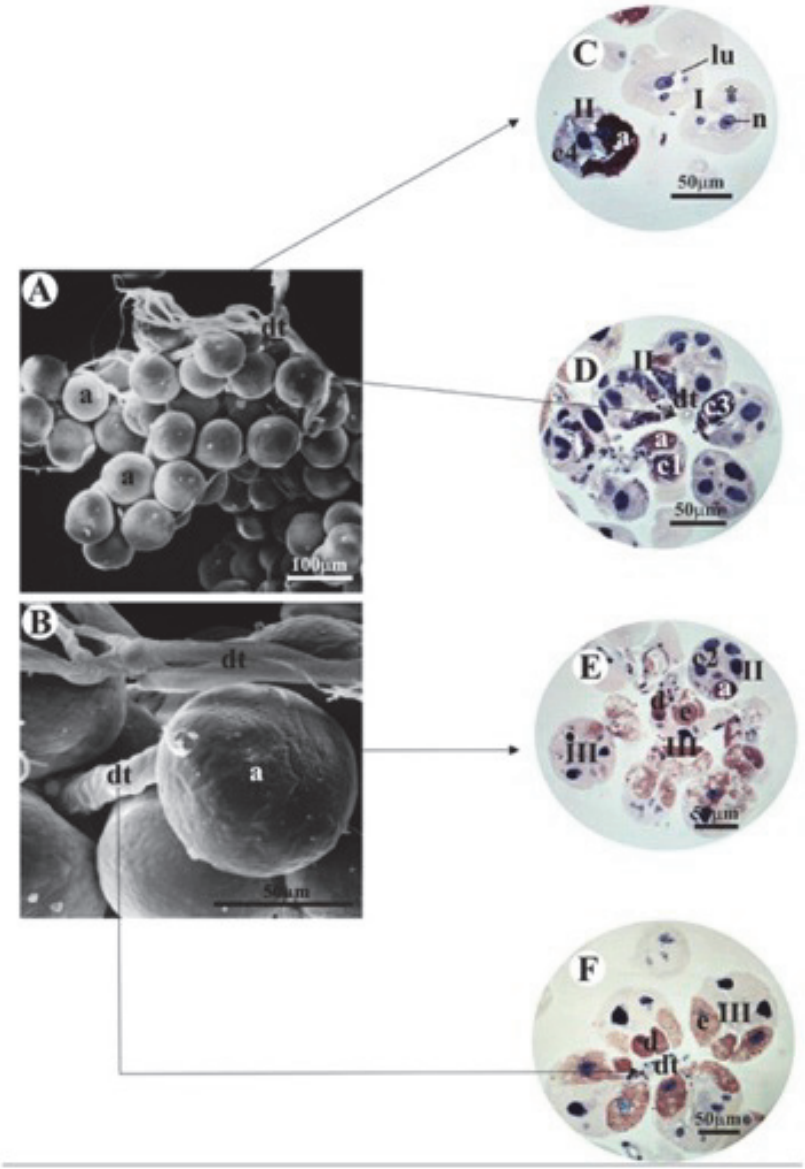


Fig. 1. (A-B) Scanning Electron Microscopy (SEM) of salivary glands of *Rhipicephalus sanguineus* semi-engorged female of the control group. (A) General view and (B) detail of the glandular acini (a) showing duct system (dt). (C-F) Histological sections of the *R. sanguineus* salivary glands of the control group stained with hematoxylin-eosin (HE) showing I (type I acinus), II (type II acinus) and III acini (type III acinus). dt = duct, n = nucleus of the central cell, * = nucleus of the peripheral cells, lu = lumen, a = a cell, c1 = c1 cell, c2 = c2 cell, c3 = c3 cell, c4 = c4 cell, d = d cell, e = e cell (Nodari et al, 2011).

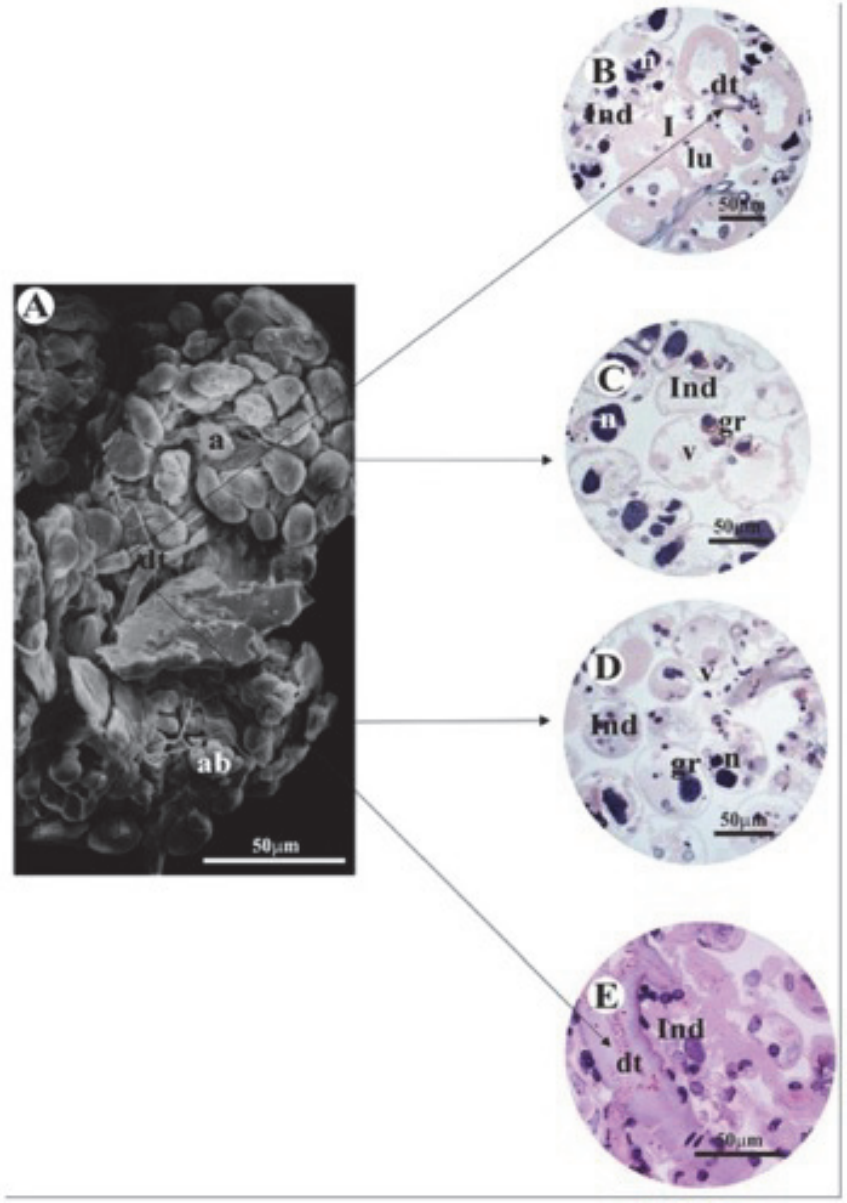


Fig. 2. (A) Scanning Electron Microscopy (SEM) of salivary glands of *Rhipicephalus sanguineus* semi-engorged females exposed to 2062 ppm of permethrin. (A) General view of the irregular acini (a), apoptotic body (ab) and ducts (dt). (B-E) Histological sections of the *R. sanguineus* salivary glands exposed to 2062 ppm of permethrin stained with hematoxylin-eosin (HE) showing I (type I acinus) with dilated lumen (lu), besides indeterminate acini (Ind). dt = duct, gr = granules, n = nucleus, v = vacuoles (Nodari et al, 2011).

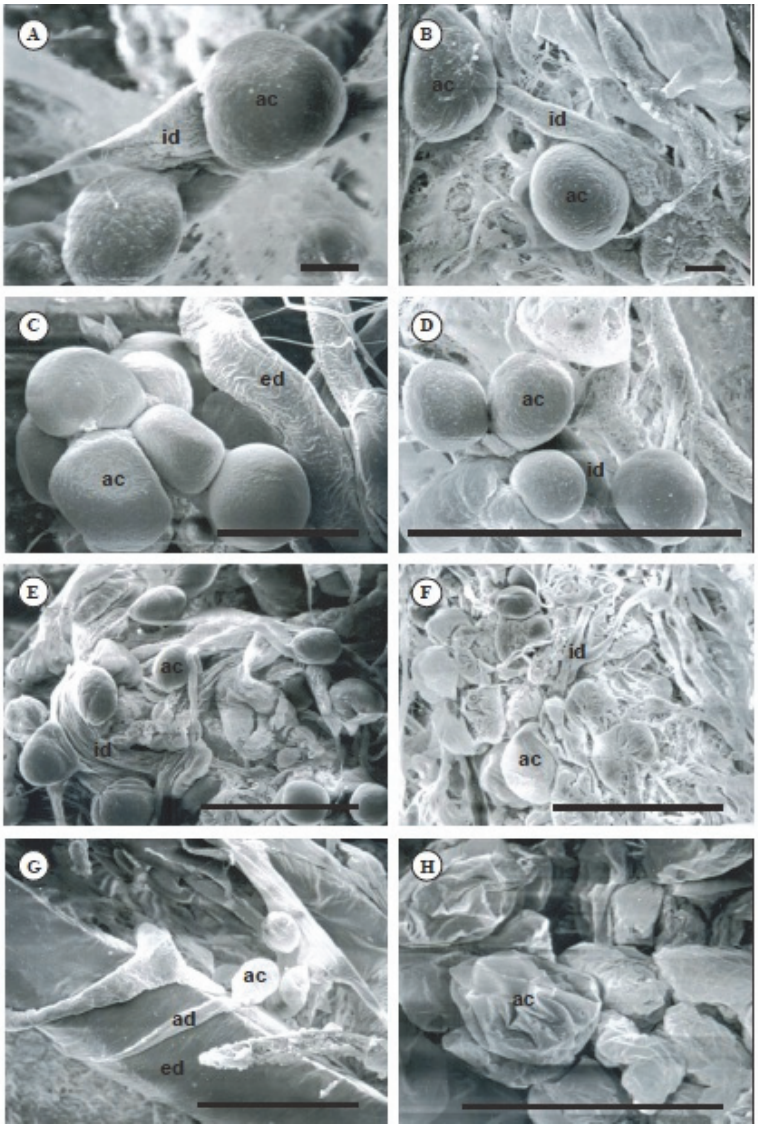


Fig. 3. Scanning Electron Micrographs (SEM) of semi-engorged females salivary glands of the tick *Rhipicephalus sanguineus* of the control group and treated with fipronil (1, 5 and 10ppm). A-B. Control group. Note in A and B, integral acini (ac) and intermediate ducts (id). C-D. Group subjected to the concentration of 1 ppm of fipronil. Less turgid acini (ac), common excretory duct (ed) and intermediate ducts (id). E-F. Group subjected to 5 ppm of fipronil. Less turgid acini (ac) and intermediate ducts (id). G-H. Group subjected to 10 ppm of fipronil. Acini (ac) with loss of turgidity or completely shriveled (shrunken) and irregular in shape, acinar duct (ad) and common excretory duct (ed) (Pereira et al, 2011). Bars: A-B: 10 μm; C-H: 100 μm.

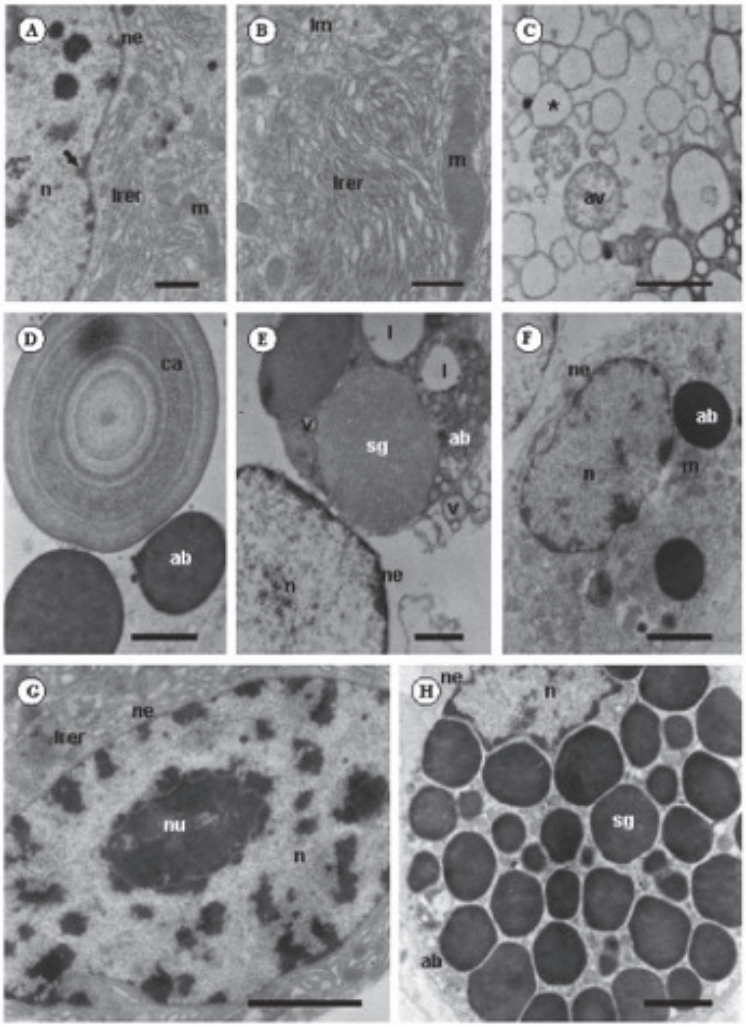


Fig. 4. Transmission Electron Micrographs (TEM) of semi-engorged females salivary glands of the tick *Rhipicephalus sanguineus* subjected to the concentration of 5 ppm of fipronil. A. Detail of the nuclei (n), marginalized chromatin (arrow) and of cytoplasm region of secretory cell with lamellar rough endoplasmic reticulum (lrer) and mitochondria (m). B. Detail of the membranous labyrinth (lm), lamellar rough endoplasmic reticulum (lrer) and mitochondria (m). C. Detail of the granular endoplasmic reticulum cisternae with dilatation (*) and autophagic vacuoles (av). D. Detail of the spherocrystals (ca) and small and homogeneous apoptotic bodies (ab). E-G. Detail of the nuclei cells (n) with nuclear envelope (ne) and marginalized chromatin. Apoptotic body (ab), lamellar rough endoplasmic reticulum (lrer), lipid (l), nucleolus (nu), proteic secretion granules (sg), vacuoles (v). H. Detail of the apoptotic body (ab) with nuclei cells (n), nuclear envelope (ne) and proteic secretion granules (sg) (Pereira et al, 2011). Bars: A, E-F = 1 μm; C-D, G-H = 2 μm; B = 10 μm.

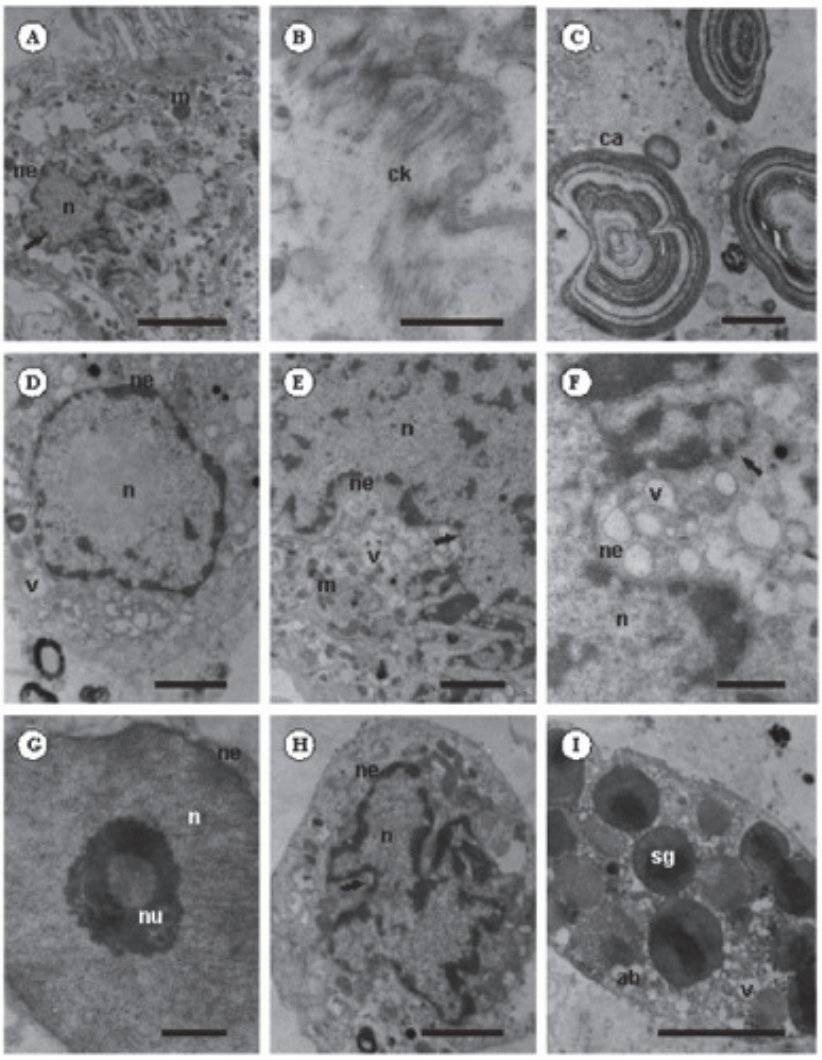


Fig. 5. Transmission Electron Micrographs (TEM) of semi-engorged females salivary glands of the tick *Rhipicephalus sanguineus* subjected to the concentration of 10 ppm of fipronil. A. Detail of completely disorganized cell where the irregular nuclei (n) with invaginations in the envelope nuclear (ne). B. Detail of the cytoplasm showing disarrange of the cytoskeleton (ck). C. Detail of the spherocrystals (ca) with completely irregular morphology. D-H. Detail of nuclei in process of cell death with invaginations (arrow) in the nuclear envelope (ne) and cytoplasmic vacuolation (v). Note in G, nuclei (n) with ring-shaped nucleolus (nu), typical characteristic of cells in cell death process. H. Apoptotic cell with irregular (arrow) nuclei (n). I. Detail of apoptotic body (ab) presenting extense process of vacuolation (v) and secretion granules (sg) (Pereira et al, 2011). Bars: B, F-G=1 μm; C-E, H = 2 μm; A, I = 5 μm.

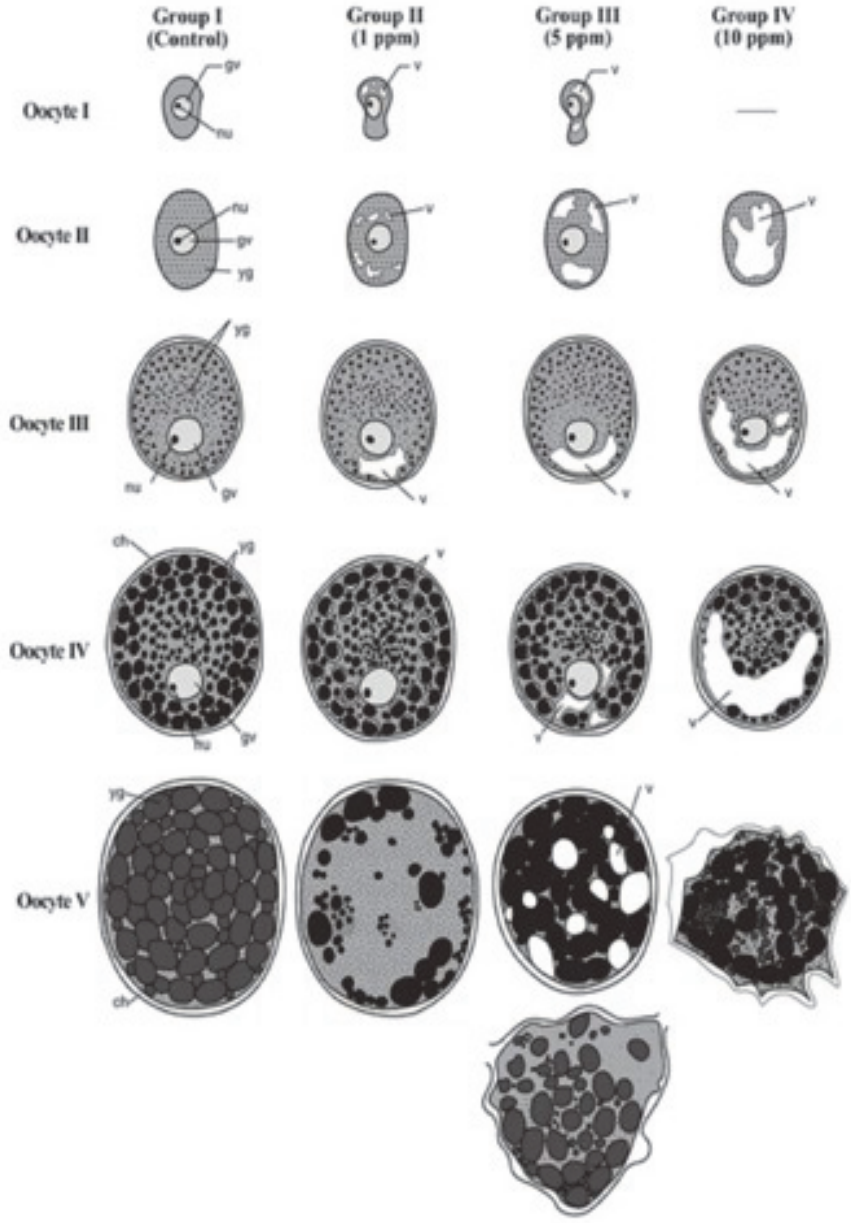


Fig. 6. Schematics drawing of the histological alterations observed in oocytes (I to V) of *Rhipicephalus sanguineus* treated with fipronil in different concentrations (control group, 1 ppm, 5 ppm, 10 ppm). ch= chorium; gv= germ vesicle; I= oocyte stage I; II= oocyte stage II; III= oocyte stage III; IV= oocyte stage IV; V= oocyte stage V; nu= nucleolus; pm = plasmic membrane; v=vacuoles; yg= yolk granules (Oliveira et al, 2008).

4. *Rhipicephalus sanguineus* ovaries

The tick ovaries in general are classified like a panoistic type; therefore it lacks nurse and follicular cells, as demonstrated in *Amblyomma cajennense* (Denardi et al, 2004), *R. sanguineus* (Oliveira et al, 2005) and *A. triste* females (Oliveira et al. 2006). It consists of a wall of epithelial cells, the pedicel, cellular structure that, besides supplying elements of the yolk to the oocytes, also connect them to the ovary wall (Till, 1961; Oliveira et al. 2005, 2006). The ovaries are usually filled with many oocytes, which will go through various developmental stages (I to V or VI) until they are ready for oviposition (Said, 1992, Saito el al. 2005).

4.1 Fipronil x *Rhipicephalus sanguineus* ovaries

Fipronil substance that acts inhibiting the development of ticks in pets animals (Taylor, 2001) and as a neurotoxic substance (Cole et al, 1993) also acts in the reproductive process of the ticks by altering both the structure and function of germinative cells of *R. sanguineus* (Oliveira et al., 2008, 2009). In these individuals ovaries the same authors detected alterations related with the size and morphology of the oocytes as well as presence of vacuoles in the cytoplasm and oocytes that had problem to succeeded in arriving at stage V (mature one), some of them had ruptured or withered, and had released their contents before the end of maturation. In females subjected to 10 ppm of fipronil (the maximal concentration tested) the oocytes in stage I were no longer observed, showing clearly that as the concentrations of fipronil increase the number and type of morphological changes in oocytes I increase as well (Fig. 6). Other data that would confirm the action of fipronil on the development of the *R. sanguineus* ovary would change the size of the oocytes, when comparing the control group to the three groups of treatment (1, 5 and 10 ppm of fipronil). The exposure of *R. sanguineus* feeding females to the fipronil leads to the susceptibility of its oocytes (in various degrees of development) to the chemical agent applied and, consequently, indicate the potential to cause damage in the reproductive process and to reduce the fertility of these ectoparasites (Fig. 7).

5. Natural acaricides

Thinking in the non-target organisms as well as in the environment preservation found alternative ticks control using biocompounds has an interesting subject with the intent to resolve some problems. In this sense is necessary to prevent the damages those are inflicted on non-target organisms, whereas the addition of financial savings the natural products could bring to producers and to the environment a sustainable management, since the environmental pollution caused by the indiscriminate use of toxic substances selects resistant animals, making the search for alternatives to natural and efficient chemicals, urgent. Plant compounds have been shown to be an important alternative for the control of these ectoparasites (Olivo et al., 2008).

5.1 Castor been oil esters x *Rhipicephalus sanguineus* salivary glands

Of the many plant species that have thus far been studied for future use in effective control of ticks is included the castor bean plant (*Ricinus communis*) which has recently emerged as a great promise, since their components such as oil extracted from it, is already widely known (biodiesel and dermatologic products). Also this plant has been supplying active principles that have enabled the fabrication of medical protheses, which besides being used in Brazil has been widely exported to the world.

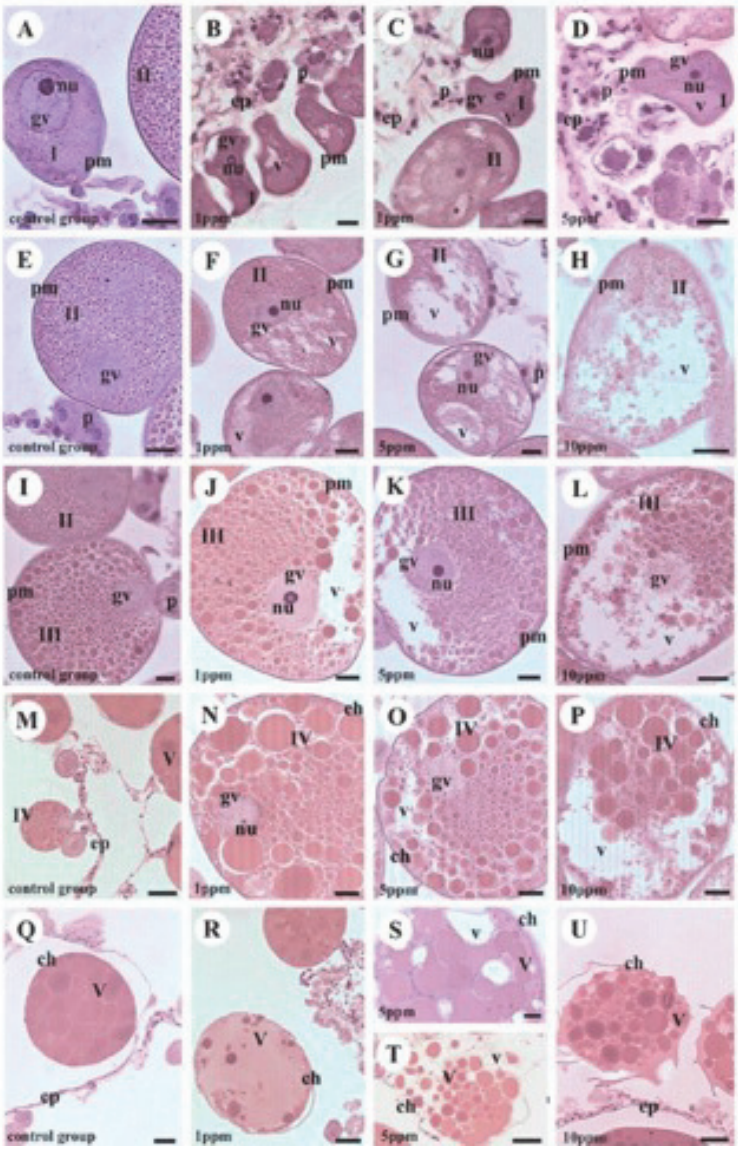


Fig. 7. Histological sections of *Rhipicephalus sanguineus* ovary stained by HE. A. E. I. M. O. Oocytes of the control group (group I) B. F. J. N. R. Oocytes of the group II (subjected to fipronil 1 ppm). C. G. K. O. S. T. Oocytes of the group III (subjected to 5 ppm). D. H. L. P. U. Oocytes of the group IV (exposed to 10 ppm). ch= chorium; ep= ovary epithelium; gv= germ vesicle; I= oocyte stage I; II= oocyte stage II; III= oocyte stage III; IV= oocyte stage IV; V= oocyte stage V; nu= nucleolus; p= pedicel; pm = plasmic membrane; v=vacuoles (Oliveira et al 2009). Bars: A-L= 0.02 mm; M= 0.1 mm; N= 0.02 mm; O= 0.02 mm; P= 0.02 mm; Q= 0.1 mm; R= 0.1 mm; S= 0.02 mm; T= 0.1 mm; U=0.05 mm.

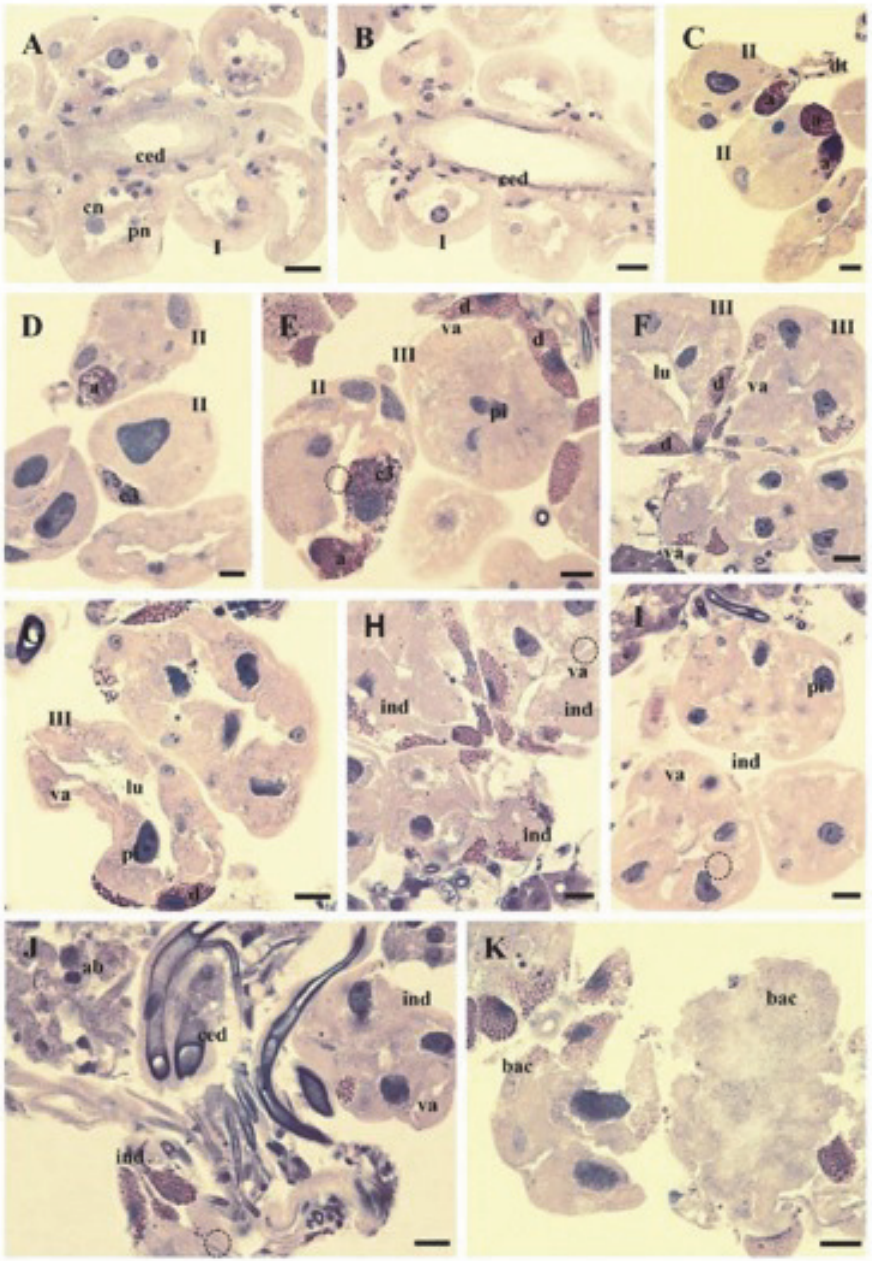


Fig. 8. Histological sections of salivary glands of engorged tick females *Rhipicephalus sanguineus* ticks (T1) stained with HE. I = acini I; II = acini II; III = acini III; ind = Indeterminate acini; a = cell a; c3 = c3 cell; d = d cell; bac = broken acini; dt = acinar duct (Arnosti et al. 2010) Bar: A–H = 20 um.

Recently a new application for the components extracted of castor plants *R. communis* has emerged from studies conducted by BCSTM (Brazilian Center of Studies on Ticks Morphology) headquartered at UNESP, Rio Claro, SP, Brazil coordinated by Profa. Dra. Maria Izabel Camargo Mathias in an attempt to offer an alternative way for tick control.
Ricinoleic acid esters from castor oil *R. communis* have been shown to efficiently in control micro and ectoparasites. Recent studies on the use of non-polluting plant the esters have shown that they act in the hydrolysis of saccharides and the dissolution of lipids in different biological systems (Mandelbaum et al., 2003). The consumption of castor oil esters by rabbits hosting the dog tick *R. sanguineus* significantly affected the vitellogenesis of the female, by making the oocytes non-viable and/or causing their death, and thus preventing new offspring from developing.
The action of the ester in salivary glands was also studied by Arnosti et al (2010a), and they showed that these esters, provided through food (feed+esters) for the rabbits hosts, enhancing and accelerated their degenerative process, that would otherwise occur slowly. It was also shown that the damage caused by esters in glandular cells was proportional to the concentration of the product, in other words at higher concentration (5g of ester (stabilized in NaCl)/Kg of commercial food for a period of 10 days. The results showed that, unlike conventional methods of chemical synthetic control, which kills ticks by neurotoxic action, different concentration of ester of *R. communis* oil did not cause the death of the ticks, but interfered in two interdependent systems, which are of paramount importance to these ectoparasites (by inducing premature degeneration of the salivary glands), and also acting in the reproductive process (preventing the vitellogenesis) (Fig. 8). Thus the action of esters on the salivary glands makes the ticks feeding process deficient causing the absence of some components in the hemolymph and consequently impairing the vitellogenic process of the oocytes (Arnosti et al., 2010a,b).
These first results performed in laboratorial assays by Arnosti et al (2010 a, b) were very promising showing the effect of this component not only in salivary glands but on the reproductive system of ticks *R. sanguineus* (reducing significantly the eggs viability), avoiding the individuals from completing the blood meal process considered necessary for effective ticks success (Fig. 9).

5.2 *Azadirachta indica* (neem plant) x *Rhipicephalus sanguineus* ovaries

In this sense, another plant of the Meliacea family (*Azadirachta indica*) or "neem" plant, have supplied actives products (extracted from leafs and seeds), which are effective to cause the reproduction damage in the ticks, by avoiding the maturation of oocytes causing consequently inhibition of the proliferation of ticks new generations. According by Denardi et al (2010) aqueous extracts obtained from leafs of neem, caused serious damages in the ovary cells of *R. sanguineus* engorged females, including morphological irregularities in the oocytes shape, changes in the yolk granulation and modifications in the germinal vesicle (oocytes nucleus) which contain the genetic material and consequently inhibing the progress of vitellogenesis, important process to reproductive success of this species (Fig. 10, 11).
Azadirachta indica would be an interesting alternative way to control ticks, since the azadiractin the active compound, can be easily obtained by management of the leafs to produce the extracts (aqueous and alcoholic), as well as saving non targets organisms once this plant is also consumed by humans in several products (like tea) in several countries in the world, meanly by Indian people.

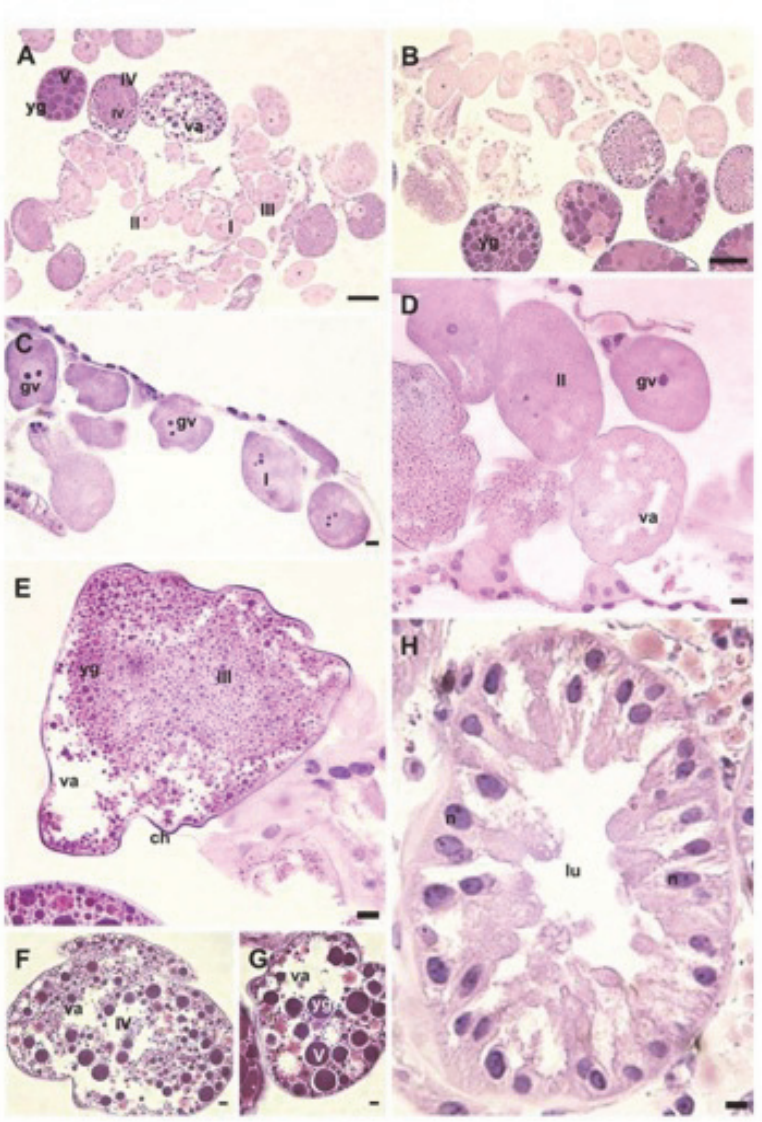


Fig. 9. Histological sections of ovary of *Rhipicephalus sanguineus* stained with HE. Treatment I with esters of *Ricinus communis* (T25g/kg food) (AB) Oocytes in various stages of maturation; vacuoles = va, yolk granules = yg; (C) Detail of oocyte I, germ vesicle = gv; (D) Details of oocytes II and III, germ vesicle = gv, vacuoles = va; (E) Detail of oocyte III, chorion = ch, vacuoles = va, yolk granules = yg; (F) Details of oocyte IV, vacuoles = va (G) Details of oocyte V, vacuoles = va, yolk granules = yg; (H) Detail of the ampole with sperm in the lumen= lu and nucleus of the ampole cell =n (Arnosti et al, 2010) Bar: A, B = 100 µm, Bar: C-H = 20 µm.

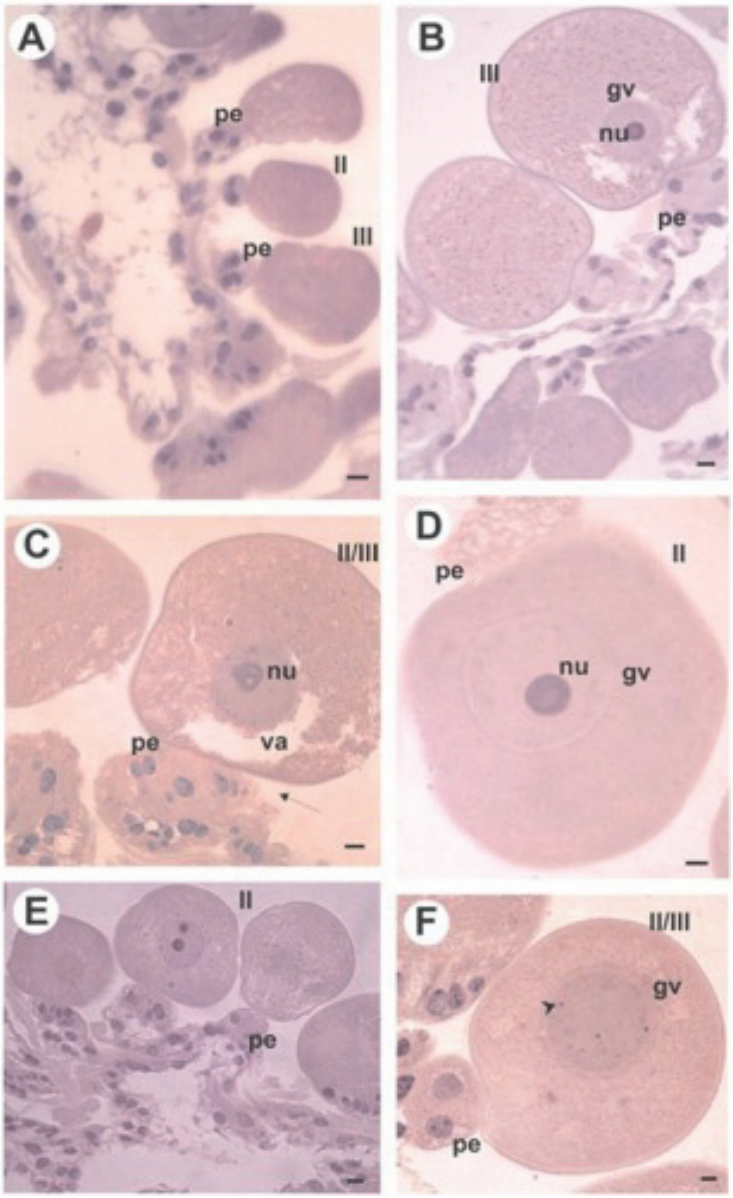


Fig. 10. Histological sections showing details of oocytes I and II, as well as the pedicel cells (pe), in the ovaries of semi-engorged females of *Rhipicephalus sanguineus* (A, C, E) and engorged ones (B, D, F) of *R. sanguineus* stained with hematoxilin and eosin (HE) (Denardi et al, 2010). Arrow = oocytes pole in contact with the pedicel cells (pe) where bigger vacuolation zone (va) can be observed. Note germinative vesicle (gv) with fragments of nucleoli (nu). Bar: 10µm A, B = control group; C, D = 10% treatment; E, F = 20% treatment.

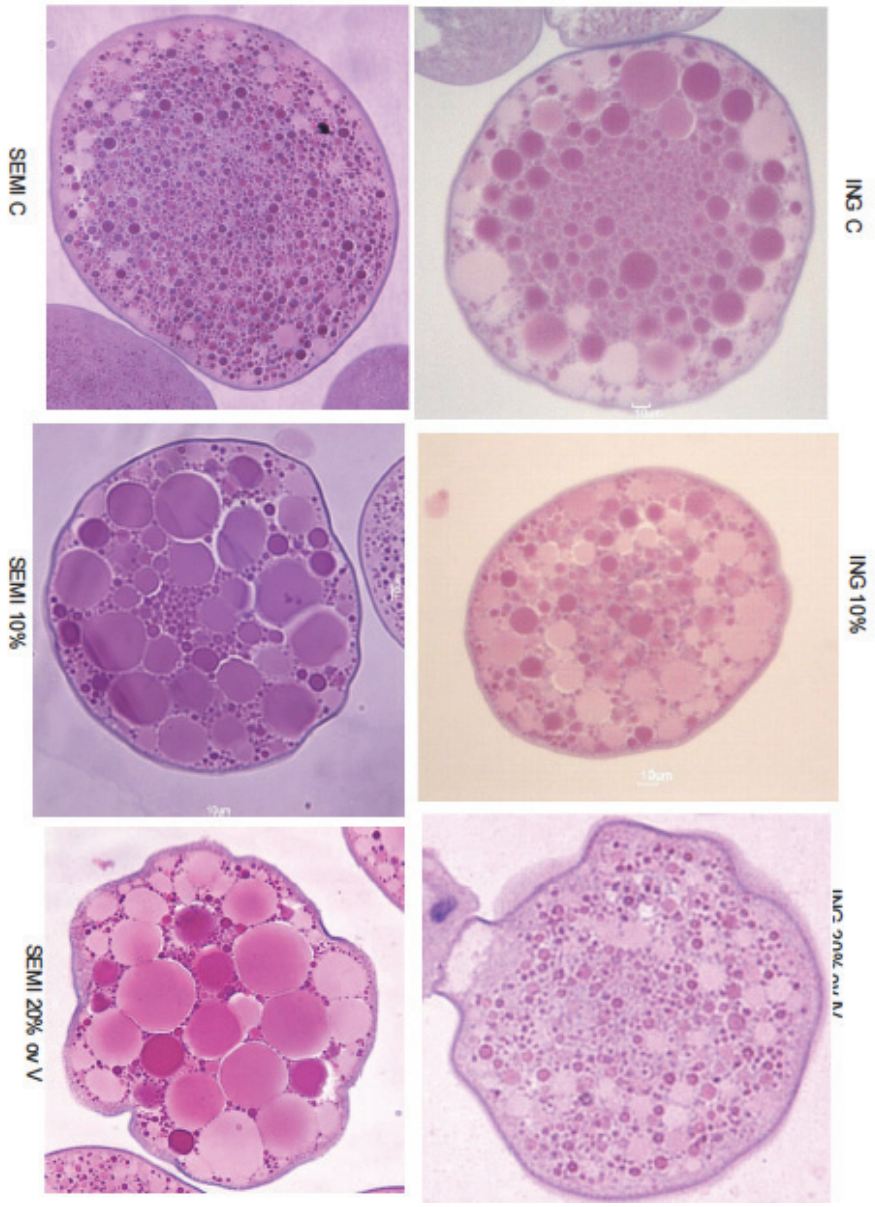


Fig. 11. Histological sections showing oocytes subjected to neem extract, in stages IV and V in the ovaries of *Rhipicephalus sanguineus* semi-engorged females (A, C, E) and engorged ones (B, D, F) stained with hematoxilin and eosin (HE), showing deformations (folds) (arrow) in the chorion (ch), and modified yolk granulation, when compared to the control group. Bar = 10µm (Denardi et al, 2010). A, B = control group; C, D = 10% treatment 10%; E, F = 20% treatment.

Besides these alternative option to control the ticks the BCSTM researcher in Brazil showed the effects of synthetic chemicals acaricides (permethrin and fipronil) in reproductive and salivary systems of *R. sanguineus* and demonstrate that lower doses and concentrations of acaricides would be used to prevent the ticks reproduction and feed, minimizing the damages to non-target individuals as well as to the environment (Roma et al., 2010, Oliveira et al. 2010).

The results presented here have also shown that doses of synthetic acaricides would be still efficient and much smaller and much less harmful to non-target organisms and to the environment being enough to reduce the harm caused by these ticks. This study confirm that chemical agents act by reducing and/or preventing the reproduction of females of *R. sanguineus* ticks, since many of the oocytes from individuals subjected to different acaricides exhibited major changes in the germ cells in relation to the control group.

Besides this, the natural compounds can be used in the management and combating of ticks within a context of sustainability of the systems, resulting in reduction of damage both to the hosts and environment (alternative tick control).

6. References

Arnosti, A., Brienza, P.D., Furquim, K.C.S., Chierice, G.O., Claro-Neto, S. Bechara, G.H., Sampieri, B.R.,. Camargo-Mathias, M. I. (2010a) Effects of *Ricinus communis* oil esters on salivary glands of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari:Ixodidae). *Experimental Parasitology*, (October, 2010) D.O.I. 10.1016/j.exppara.2010.10.009, 2010ISSN: 0014-4894

Arnosti, A., Brienza, P.D., Furquim, K.C.S., Chierice, G.O., Claro-Neto, S. Bechara, G.H., Sampieri, B.R., Camargo-Mathias, M.I. (2010b). Effects of ricinoleic acid esters on the vitellogenesis of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari:Ixodidae) tick. *Experimental Parasitology*, (October, 2010) D.O.I. ISSN: 0014-4894

Cole, L.M., Nicholson, R.A., Casida, J.E. 1993. Action of phenylpyrazole insecticides at the GABA-gated chloride channel. *Pesticide and Biochemistry Physiology*, 46-47-54. ISSN: 0048-3575

Denardi S.E., Bechara G.H., Oliveira P.R., Nunes, E.T., Saito, K.C., Camargo-Mathias, M.I. (2004) Morphological characterization of the ovary and vitellogenesis dynamics in the tick *Amblyomma cajennense* (Acari: Ixodidae). *Veterinary Parasitology* (July, 2004) 125:379-395, DOI:10.1016/j.exppara.2010.05.017. ISSN: 0304-4017

Denardi S.E., Bechara G.H., Oliveira P.R., Camargo-Mathias, M.I. (2010) *Azadirachta indica* A. Juss (neem) induced morphological changes on oocytes of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) tick females. *Experimental Parasitology* (November, 2010) DOI:10.1016/j.exppara.2010.05.017. IssnISSN: 0014-4894

Denny, D.J.B. 2001. Efficacy of fipronil against ticks. *Veterinary Recherche* 148-124

Furquim, K.C.S., Bechara, G.H., Camargo-Mathias, M.I. 2007. Cytoplasmatic RNA and nuclear changes detected cytochemically during the degeneration of salivar glands of the tick *Rhipicephalus sanguineus* (Latreille, 1806) (Acari:Ixodidae*) Micron* (November, 2010) 39:960-966. ISSN: 0968-4328.

Furquim, K.C.S., Bechara, G.H., Camargo-Mathias, M.I. 2008. Death by apoptosis in salivary glands of the tick *Rhipicephalus sanguineus* (Latreille, 1806) (Acari:Ixodidae). *Experimental Parasitology* (February, 2008) 39:960-966.ISSN 0014-4894

Garcia-Garcia, E., Bussacos, M.A., Gisher, F.M. 2005. Impacto da legislação no registro de agrotóxicos de maior toxicidade no Brasil. *Revista de Saúde Pública* 39:832-839. ISSN 0034-8910.

Higgins, R.A., Sloderbeck, P.E. Brooks, H.L. 2001. *Field corns insect management for 2001*. Kansas: Kansas State University, 18-24.

Hugnet, C., Cadore, J.L., Bourdoiseau, G. 1999. Use of fipronil spray (0,25%) for the treatment of *Damlinia equi (Wereckiella equi)* infestation. *Pratique Veterinaire Equine* 31:65-68. ISSN:0395-7500.

Mandelbaunm, S.H., Santos, E.P., Mandelbaum, M.H.S. 2003. Cicatrização: conceitos atuais e recursos auxiliares- Parte II. *Anais Brasileiros de Dermatologia* 78:525-542. ISSN 0365-0596.

Mencke, N., Volp, P., Volfova, V., Stanneck, d. 2003. Reppelent efficacy of a combination containing imidaclorprid and permethrin against sand flies (*Phlebotomus papatasi*) on dogs. *Parasitology Research* 90:108-111. ISSN: 1432-1955.

Moorhouse, D.E., Tatchell, R. 1966. The feeding processes of the cattle tick *Boophilus microplus* (Canestrini): a study in host-parasite relations. Part I. Attachment to the host. *Parasitology* 56:623-632. ISSN: 0031-1820 EISSN: 1469-8161.

Nodari, E.F., Roma, G.C., Furquim, K.C.S., Bechara, G.H., Camargo-Mathias, M.I. 2001. Cytotoxic effects of permethrin in salivary glands of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari:Ixodidade) semi-engorged females. *Experimental Parasitology* (February, 2011) DOI 10.1016/j.exppara.2011.02.003. ISSN: 0014-4894

Nuttal, G.H.F, Strickland, C. 1908. On the presence of an anticoagulin in the salivary glands and intestines of *Argas persicus. Parasitology* 1:300-310. ISSN: 0031-1820 EISSN: 1469-8161.

Oliveira P.R., Bechara G.H., Denardi S.E., Nunes E.T., Camargo-Mathias, M.I. (2005) Morphological characterization of the ovary and oocytes vitellogenesis of the ticks *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae). *Experimental Parasitology* (March, 2005) 110: 146-156. . ISSN: 0014-4894

Oliveira P.R., Camargo-Mathias, M.I.,Bechara G.H. (2006) *Amblyomma triste* (Koch, 1844) (Acari: Ixodidae): Morphological description of te ovary and of vitellogenesis. *Experimental Parasitology* (March, 2006) 113: 179-185. .ISSN: 0014-4894

Oliveira P.R., Bechara G.H, Camargo-Mathias M.I. (2008) Evaluation of cytotoxic effects of fipronil on ovaries of semi-engorged *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) tick female. *Food and Chemical Toxicology* (March, 2008) 46:2459-2465. . ISSN: 0278-6915

Oliveira P.R., Bechara G.H, Camargo-Mathias M.I. (2009) Action of the chemical agent fipronil on the reproductive process of semi-engorged females of the tick *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae). Ultrastructural evaluation of ovary cells. *Food and Chemical Toxicology* (February, 2009) 47:1255-1264. ISSN: 0278-6915.

Oliveira P.R., Bechara G.H, Denardi, S.E., Oliveira, R.J., Camargo-Mathias M.I. (2010) Citotoxicity of fipronil in mouse liver cells. *Veterinary Research* (February, 2009) DOI 10.1016/j.fct.2009.02.019. ISSN (Electronic Edition): 1297-9716

Oliveira P.R., Bechara G.H, Denardi, S.E., Pizano, M.A., Camargo-Mathias M.I. (2011) Toxicity effect of acaricide fipronil in semi-engorged females of the tick *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae). Preliminary determination of the minimum lethal concentration and LC50. *Experimental Parasitology* (November, 2010) 127:418-422. ISSN: 0014-4894

Olivo, C.J., Madruga de Carvalho, N., Sousa da Silva, J.H., Flores Vogel, F., Massariol, P., Meinerz, G., Agnolin, C., Farias Morel, A., Volnei Viau, L. 2008. Óleo de citronela no controle de carrapatos de bovinos. *Ciencia Rural* 38:406-410. ISSN 0103-8478

Paesen, G.C. Adams,m P.L. , Harlos, K. Nuttall, P.A., Stuart, D.I. 1999. Tick histamine-binding proteins: isolation, cloning and three-dimensional structure. *Mollecular Cell* 3:661-671. ISSN 1097-2765.

Penaliggon, J. 1997. Getting to grips with fleas on pet dogs and cats. *Pesticide Outlook* 8:19-23. ISSN (electronic): 1465-8933.

Pereira, C.P.M, Oliveira P.R., Furquim, K.C.S., Bechara G.H, Camargo-Mathias M.I. (2011) Fipronil-induced cell death in salivary glands of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) semi-engorged female. *Experimental Parasitology*,(October, 2010) 127:481-489 ISSN: 0014-4894

Pereira, C.P.M, Oliveira P.R., Furquim, K.C.S., Bechara G.H, Camargo-Mathias M.I. (2009) Effects of fipronil (active ingredient of Frontline) on salivary glands of *Rhipicephalus sanguineus* females (Latreille, 1806) (Acari: Ixodidae) semi-engorged female. *Veterinary Parasitology* (November, 2009) 166:124-130 . ISSN: 0304-4017

Roma G.C., Oliveira, P.R., Pizano, M.A., Camargo-Mathias M.I. (2009) Determination of LC50 of permethrin acaricide in semi-engorged females of the tick *Rhipicephalus sanguineus* (Acari: Ixodidae). E*xperimental Parasitology* (August, 2009) DOI 10.1016/j.exppara. 2009.08.001. ISSN: 0014-4894

Roma G.C., Furquim K.C.S., Bechara G.H., Camargo-Mathias M.I. (2010) Permethrin-induced morphological changes in oocytes of *Rhipicephalus sanguineus* (Acari: Ixodidae) semi-engorged females. *Food and Chemical Toxicology* (May, 2010) 48:825-830. ISSN: 0278-6915

Saito, K. C., Bechara, G.H., Nunes, E.T., Camargo-Mathias, M.I. 2005. Morphological, histological and ultrastructural studies of the ovary of the cattle tick *Boophilus microplus* (Canestrini, 1887) (Acari:Ixodidae). *Veterinary Parasitology* (September, 2004) 129:299-311. ISSN: 0304-4017

Sonenshine, D.E. 1991 The female reproductive system. In: Sonenshine, D.E. (Ed.) Biology of ticks. Oxford University Press. New York, 280-304.

Taylor, M.A. 2001. Recent developments in ectoparasiticides. Veterinary Journal, 161:253-268.

Tellam, R.L., Smith, D., Kemp, D.H., 1992. Vaccination against ticks. In:Young, W.K. (Ed.), Animal Parasite /control Using Biotechnology. CRC Press, Boca Raton, pp. 303-331.

Walker, A., Gletcher, J.d., Gill, H.S. 1985. Structural and histochemical changes in the salivary glands of *Rhipicephalus appendiculatus* during feeding. *International Journal for Parasitology* 15(1), 81-100.

Willadsen, P. 1997. Novel vaccines for ectoparasites. *Veterinary Parasitology* 71:209-222. ISSN: 0304-4017

18

Neem Tree (*Azadirachta indica* A. Juss) as Source of Bioinsectides

Marcello Nicoletti[1], Oliviero Maccioni[2],
Tiziana Coccioletti[2], Susanna Mariani[2] and Fabio Vitali[2]
[1]*Department of Environmental Biology - University Sapienza of Rome, Roma,*
[2]*ENEA C.R. Casaccia – S. Maria di Galeria (Roma),*
Italy

1. Introduction

The interest in developing pesticides of natural origins has increased during recent years, because of the drawback of synthetic chemical pesticides, like impact on environment, toxicity to non target organisms including humans, resistance development in insect population. Furthermore, other considerations emerged, including low cost, renewable raw material, like wasting, interest for a possible individual use in urban area. In this paper attention was focused on products of neem tree, *Azadiractha indica* (A. Juss, 1830), and in particular on neem cake, the by-product obtained in the cold-pressed process of neem oil production. Actually neem cake marketed products are not used as pesticides, but mainly as fertilizers. Data on their compositions and insecticidal activity are lacking.
Studies on marketed neem cake products made by HPTLC and HPLC analyses showed differences in their compositions, in particular on limonoids. Once determined limonoid contents in extracts of increasing polarity, these contents were related to larvicidal activity on Asian tiger mosquito, *Aedes albopictus* (Skuse, 1894). The aim of the reported studies is check the possibility of developing a new domestic insecticide using neem cake as raw material, in particular against biting mosquitos present in urban areas.

2. Micro vs. macro competing for life

Microorganims are difficult to find and to kill, because they are in enormous number and everywhere. For instance, in our body we can count more procariotic than eucariotic cells. Fortunately, most of them are useful friends, but others can be very dangerous and destructives.
Actually, microorganisms are liable of major plagues affecting humans. These invisible our competitors act infections by complicated mechanisms, often involving other creatures. Mosquitos are the favorite partners as major vector of transmission. Therefore, mosquitos are co-responsible of malaria, dengue fever, yellow fever, filariasis, schistosomiasis, Japanese encephalitis, Chagas morbus, hemorrhagic fever, arbovirosis, as well as of several minor pathologies, such as systemic allergic and inflammatory reactions and dolorous bites. Although in developed countries the impact of these pathologies is nowadays restricted or absent, and main causes of death are related to physiological aspects (cancer, hearth and

coronary failures, ecc.), in the remaining predominant part of the world, the alert is always the same and means infection by injure or by bite.
Practically all mankind living in ordinary conditions is continuously exposed to one or more mosquito-borne or connected diseases and suffer in different degrees the effects of the mosquitos attack. Only dengue worldwide threatens the health of around 2.5 billion people, and figures for malaria are surely worst. Malaria infects more than 500 million humans each year. About 90% of cases occur in Africa, including those of malaria-related deaths, but only in India 15 million cases and 20,000 deaths are estimated annually by WHO.
However, as all living beings, also microorganisms have their own Achilles heel. Their movement capacity is very limited, therefore they use animals as vectors for efficiently diffusing in every habitats. Usually, they change to adapt to the host, accumulating therein and becoming vulnerable. Therefore, the option seems to be simple: kill the vector and kill the microorganism.

3. Fighting the vector

Several strategies have been proposed against microorganism/mosquito based diseases in order to control or at least limit mosquitos invasion, mainly based on three types of action: direct, environmental, indirect. Direct methods use as target the adults, whereas indirect methods are mainly focused on effects on mosquito development, including controls of larvae by hormones or other growth regulators. Environmental methods are based on change in the habitat of the insect and display severe collateral effects on other organisms.
So far, mainly synthetic insecticides have been produced and used, in large quantities and types. Initial euphoria for the resolving effects has been punctually followed by negative drawback. Chemical pesticides resulted non-selective, that means harmful and toxic to other organisms including humans, plus the cause of a series of unexpected and durable environmental damages. Several challenges continue to hinder efforts to effectively control vectors, including the induction of insect resistance, insect adaptation and altered behavioral traits, such as exophily and exophagy, not considering limited resources that affect conventional use of control methods in so many countries. The main problem was their inefficacy during the time, leading to the urgent need for novel effective insecticides, rising from natural products.

4. Focusing on larvicides

Generally, interest on biopesticides followed that in natural products applications. Botanical insecticides started in the early 1930s and continued to the 1950s, but it was eclipsed when synthetic insecticides appeared on the scene. However, during the last two decades a revival of natural products overbore in all markets, in coincidence with the difficulties in using synthetic insecticides. Among the important and decisive struggle against microorganism/mosquito based ailments, vector control is actually considered the most feasible way, meeting the modern criteria of use in integrated pest management programs. However, current vector control methods are based on target the adult in order to reduce the vectorial capacity. These control methods must consider behavioral changes of adult mosquitos that can reduce the effect. Nowadays, for these reasons research was focused on larval control, as an overlooked method, better to be used in an integrated vector management program, that means that the environmental care is particularly noteworthy.

However, as well known, indiscriminate methods of applications, larger and unnecessary quantities and concentrations of pesticides are rampant and difficult to limit, often allowing high loads of xenobiotics to reach the soil matrix and accumulate. Therefore, concerns about chemical insecticides and their persistence effects on the environment, as evidenced by the paradigmatic case of DDT (Mulla and Su, 1999), as well as development of physiological resistance by the insects, have stimulated the search of new ecofriendly products. This also in line with 1997 World Health Assembly resolution 50.13, section 2.4 (WHO, 2004; WHO, 2005). The interest in developing bioinsecticides increased dramatically during the recent years, because of the frequent use of synthetic products in urban areas with increasing concern for toxic effects on humans and pets. Furthermore, the invasion in Europe of new more aggressive species was registered. As observed, urban habitat generates in some species effects in terms of increases of pupae production that the "traditional" ones and also complicates the use of chemical insecticides.

Nowadays, attention drove to natural substances produced by plants. A botanical insecticide should be: ecofriendly, biodegradable, target-specific against mosquito vectors. Furthermore, requisites for its market success could be: low cost, availability, easy utilization and simple storage.

As a paradox, the problem consists in the excess of the offer, i.e. the quantity of plants and natural products as possible real candidates. It is estimated that around 100 000 secondary metabolites have been isolated, mainly from plants, but the total number should be much higher, being only angiosperms more that 330 000 species, wherein only the 10% has been the object of relevant phytochemical interest. So far, at least more that 2000 plant species reportedly possess potential for pest control and several are already present in the market. Only in the family of Meliaceae six species have been studied for pesticidal properties (Mulla and Su, 1999) and at least 35 biologically active compounds identified. In any case, random studies are costly and complicated and a strategy of selection must be constructed.

5. Selecting botanical candidates

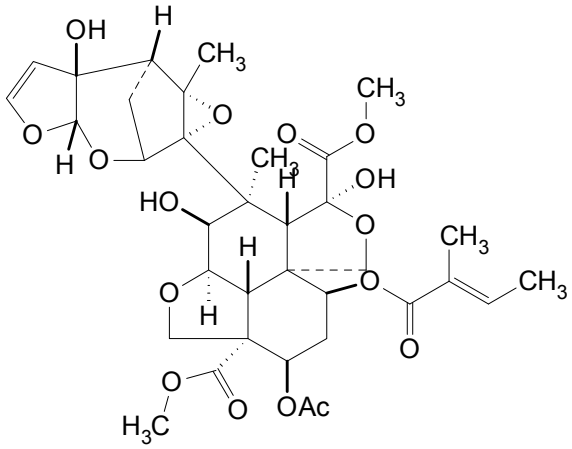
As the history of medicine teaches, information derived from traditional uses can be very useful. Among the plants so far selected as best candidates for developing of natural insecticides, the neem tree, *Azadirachta indica* A. Juss, has already gained a special place, with its reported activity against 400 insect pests. Among the Meliaceae, the Mohogamy family, the genus *Azadirachta* consists of few species, the most important being *A. indica*, a moderate to large tree. It easily and rapidly grows, reaching 80 cm in one year and survives even on dry, nutrient-lean soils (Pundy, 2000). Native to the Indian subcontinent, where is normally found, from Uttar Preadesh to Tamil Nadu, neem continuously spreads in the world. Being a very valuable forestry species, as a multipurpose tree, it is considered ideal for reforestation programs and is largely cultivated world widely, including Central and South America, several countries of Africa and several parts of Asia, like China and Vietnam. Several varieties are also reported, like in Thailand, the *A. indica* var. *sinensis*, locally called the Sadao tree. Limitations concern the preference for tropical and subtropical areas and altitudes not higher that 1000 m. It is often confused with the Indian Lilac, *Melia azedarach*, also known as Chinaberry tree or cinnamon or Santa Barbara in Brazil.

6. Using neem tree

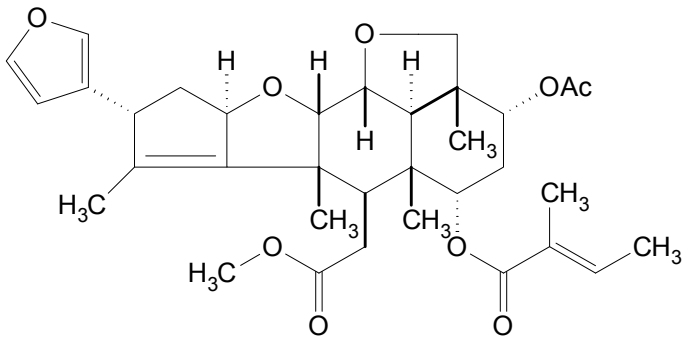
The International Scientific Community, given the enormous amount of results that validate the medicinal properties of Neem, includes neem tree into the top ten list of plants to be studied and used for the sustainable development of the planet and the health of living beings. Neem, identified by WHO/UNEP1989 as an environmentally powerful natural pesticide, is considered to be one of the most promising trees of the 21st century for its great potential in pest management, environment protection and medicine. In this case, sustainability is very high. Not considering that commercial uses are actually restricted the use of removable parts, like leaves, fruits and seeds, there are about 14 million neem trees growing only in India and the plant is adapted to subarid and subhumid areas of tropical and subtropical areas. Indian trees have the potential to produce 3.5 million tonnes of seed/year, corresponding to a production of 700,000 tonnes of oil/year. Neem is well known and used for its medicinal properties from the ancient period (4000 BC); mainly on the indications of Ayurveda medicine, being very popular, even revered in the Indian Subcontinent (Gajalakshimi S. and Abbasi S.A., 2004). In practice, all parts are traditionally used for a variety of indications, but limiting to the ethnobotanical indications concerning the aim of this paper, we can recall the use of neem in indigenous medicine as a bitter tonic, antimalarial, antipyretic, anti-inflammatory, antihelmintic, and for antimicrobial and antiviral effects (Varie, 1996). Fruits are collected when the drupes turn yellowish-green by hands or machines and are processed as soon as possible. The fleshy part of the fruit is removed and the stones washed in clean water and dried for 5-10 days. The oil is obtained usually by large mechanical expellers or by solvent extraction, only small-scale producers still use traditional pressing methods.

The seeds contain about 45% of a brownish-yellow of fixed oil, mainly constituted by oleic acid (50-60%), palmitic acid (15-19%), stearic acid (14-19%), linoleic acid (8-16%) and characterized by an acrid taste and a persistent and unpleasant odour (Mongkholkhajornsilp et al., 2005). The essential oil reported hexadecanoic acid (34.0%), oleic acid (15.7), 5,6-dihydro-2,4,6-triethyl-(4H)-1,3,5-dithiazine (11.7), methyl oleate (3.8), and eudesm-7(11)-en-ol, as determined by GC-MS (Kurose and Yatagai, 2005). There are also many other constituents reported, i.e. pigments, polisaccharides, salts and the proteinaceous material that makes up the cellular matrix of the seeds (Johnson & Morgan,1997). However, the quality of the oil is highly affected by the method of processing, as well as the types of seeds. More than 300 compounds have been characterized from neem seeds, with over 50 different bioactive constituents from various parts of neem tree. One-third of reported constituents belong to the class of nortriterpenes, and more preciously to the steroidlike tetranortriterpenoids, named limonoids. Neem limonoids belong to nine basic structures: azadiron (from seed oil), amoorastatin (from fresh leaves), gedunins (from kernels), salannin (from fresh leaves and seeds), and azadirachtin (from seed oil). Azadirachtins (from A up to H), highly oxygenated C-secomeliacinlike compounds, are the predominant and the most studied, including azadirachtin A (AzA), AzB, nimbin, salannin as the most important (Silva et al., 2007 and reference therein), any single main constituent is present with a series of derivatives, i.e. nimbim, nimbinin, nimbidin and nimbidiol. Several marked differences have been reported in the yield of limonoids neem seeds due to geographical origins or even by collection in different seasons in the same area. AzA usually resulted the prevalent constituent followed by AzB (3-tigloylazadirachtol) as second, with concentration up to 15% of the total Az. The different formulations of neem expeller oil have a content of azadiracthin from 300 to 2000 ppm. (http://www.agroextracts.com/).

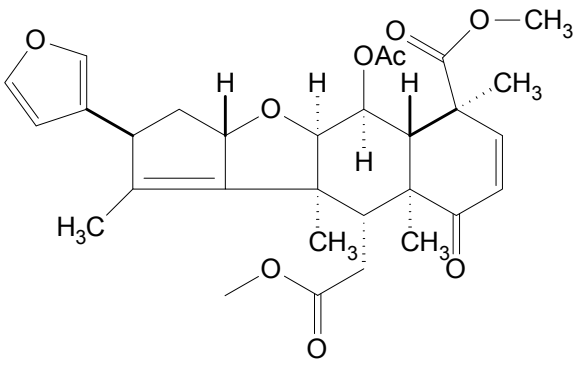
Like most of nortriterpenoids, limonoids are exceedingly bitter. They have attracted global attention for their insecticidal, fungicidal and nemicidal properties (Gajalakshmi and Abbasi, 2004 and references therein).



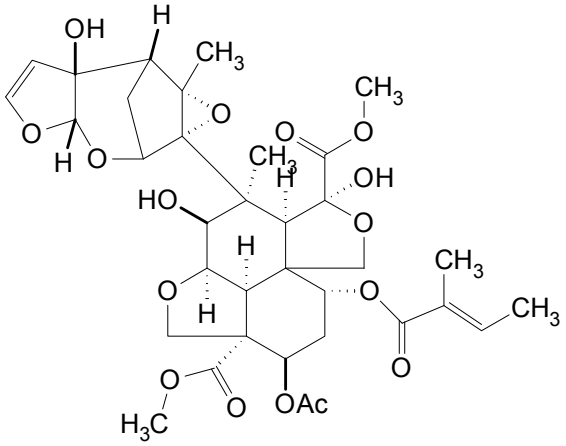
azadirachtin A



salannin



nimbin



azadirachtin B

The insecticidal properties of neem products were first reported by Chopra in 1928. Effects of neem products against mosquitos are well known and documented, both at research level and practical uses. A list of tested arthropods, at different growing stated up to 1993 can be found in paper of Mulla and Su (Mulla and Su, 1999). In particular, it protects against the bite of *Anopheles*, it repels *Culex quinquefasciatus* (Su and Mulla, 1999) and *Aedes* spp., it causes nymphal death in *Bamisia tabaci*. Larvicidal action was reported on the dengue mosquito *Aedes aegypty* (Ndione et al., 2007), the malaria vector, *Anopheles gambiae* (Okumu F.O. et al., 2007), and the filarial vector, *Culex quinquefasciatus*. These actions are based on multiactions against insects: toxicity, antimitotic effects, antifeedant activity, growth regulation, fecundity suppression, sterilization, oviposition repellency, including harmful effects on endocrinien system and damages of the cuticle of larvae preventing them from moulting (Mulla and Su, 1999). Also aqueous extracts of neem wood and bark chippings showed larvicidal activity against *A. gambiae* (Howard, 2009); in this extracts HPLC analysis

showed the presence of a series of constituents of varying polarity, including nimbin and salannin, whereas AzA was not detected. Structure complexity of Az and other bioactive neem constituents precludes any large-scale chemical synthesis by the extremely high cost. Therefore, the only practical option is the extraction from renewable parts of the tree, i.e. leaves and seeds, and manufacture various pesticidal formulations.

Neem preparations that resulted effective against insecticide-resistant pests, yet does not harm heavily the beneficial insects. Out of date, neem products are considered relatively safe to mammals and humans (Boeke et al., 2004). Anyway, the absence of toxicity is largely tested by the prominent role of fruits, bark and leaves in Indian Traditional Medicine, as well as the use of flowering and leaves as vegetables in Asian countries and the current use in a number of toiletry and pharmaceutical products. Acute toxicity of neem oil has been studied and is reported as 12 ml/Kg for rats and 24 ml/Kg for rabbits (Gandhi, 1988). General cautions must be taken for the possible presence of aflatoxins, usually present in case of bad storage, as in other botanical raw materials.

The results showed that neem products could act primarily as larvicides (Zebitz, 1986, 1987; Naqvi et al., 1991; Rao et al., 1992, 1995; Amorose, 1995; Wandscheer et al., 2004; Okumu et al., 2007, Howard et al., 2009). Organic solvent extracts and oils from neem and *Melia azedrach* have displayed several bioactivities against insects governing chemical maturation of molt hormones, chitin biosynthesis and field deterrence. Activities were referred to limonoids, i.e. Az from neem and meliacarpinin from *M. azedarach*.

7. Using neem cake

Procedure for obtaining neem cake oiled and deoiled is summarized in Fig.1. However, the extraction processes are subjected to several changes, depending the producer.

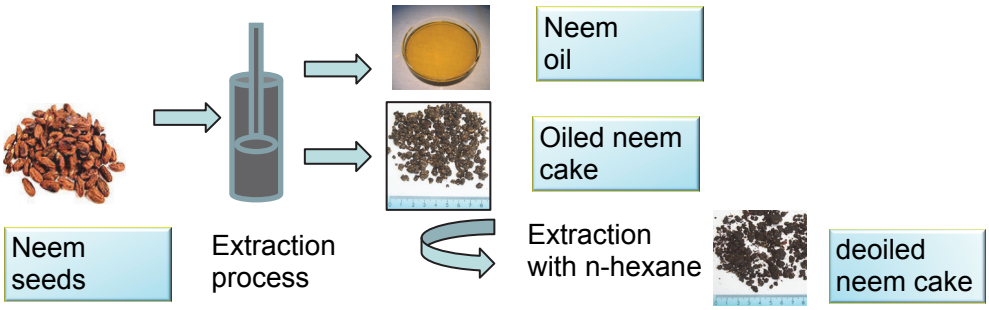


Fig. 1. The principal steps leading to the production of deoiled neem cake.

The commercialization of neem cake, usually from India, in the European market is already a reality as is allowed its use as fertilizer in organic farming and organic livestock feed supplement, in accordance with current legislation. For now, the control of product quality, only provides a label stating the contents of N, K, P and information on the type of extraction processes by which the cake comes (cold-pressed or cold-pressed, followed by extraction with organic solvent). The purpose of this study is focused on demonstrating how

the technology HPTLC can be as useful for the labeling of the product. The neem oil cake is the by-product obtained in the cold-pressed process has an oil content of about 6%, while the neem deoiled cake, the by-product obtained in the organic solvent extraction process, has still an oil content of about 1.5 %. The only present utilization of neem cake concerns its use as a natural and environmental friendly fertilizer, soil conditioner, nitrogen saver and manure in farming and agriculture (Gopal et al., 2007). Deoiled neem cake is traditionally used as fertilizer by Indian farmers, which appreciate also the pest repellent effects. When neem cakes is ploughed into the soil, it also protects plant roots from nematodes and white ants. The ethnobotanical indications were fully confirmed by recent studies, showing that neem cake at the same time acts as a pesticide and provides the much necessary nutrition to the soil microbes, besides improving the soil physico-chemical properties.

Neem cake seems to be a good pretender for developing new botanical insecticides. Beside the already mentioned qualities, we must stress on the global interest in recycle and intelligent utilization of materials considered a waste or exhausted. Moreover, it is also clear that such projects need a lot of technology, research and initial funding, but sometimes results pay the expectative of long years of studies and tentatives. For instance, a constant increase of potential yield of main crops is urgently requested, calling for massive quantities of fertilizers and pesticides. Appropriate technologies, including the use of low cost natural products should have key role to environmental safeguard. Another crucial aspect concerns the use of neem cake as agro-industrial by-product for livestock feed, as several researches and applications have suggested (Verna et al., 1995; Rao et al., 2003). Neem-cake is currently used as organic fertilizer and as feed supplement in animal husbandry. India alone has an annual potential of 80,000 tons of oil and 330,000 tonnes of neem-cake from 14 million plants that grow naturally. Neem cake is therefore a product of low cost and widely available on the world market. In particular, starting from the neem cake has been isolated a phytoextract that has a remarkably high insecticidal activity against larvae of *Aedes albopictus*, commonly known as tiger mosquitoes (Nicoletti 2010).

8. Looking for the ideal insecticide

The raw material to be used for a domestic insecticide should be:

- low cost and abundant
- no toxic itself or by processing except to the target
- composition must be determined as deep as possible and the used product stable in determined composition
- biological activity reported in details and tailored for target organism.

Our research was focused on the last two items, and *Aedes albopictus* selected a target organism owing its increasing negative role in Europe, and in particular in Italy. In the last decades, the spreading of Asian tiger mosquito, all around the world, has caused mainly the colonization of towns environment. This blood sucking mosquito raised serious concern, because its bites cause great trouble and it is a competent vector for the transmission of at least 22 arbovirus (Gratz, 2004). As a matter of fact, Asian tiger mosquito was absent in Italy since two decades ago, but after its introduction it rapidly became dominant, leaving other competitors to a secondary role. So far, adopted methods by Municipalities resulted deprived of real efficacy. Local transmission of the *Alphavirus chikungunya* (CHIK) has been referred in two small towns in the province of Ravenna,

Italy during the 2007 summer (Vazeille et al., 2007) At the present, the most spread chemical insecticides used as mosquitoes larvicidae includes organophosphates, pyrethroids and insect growth regulators. In spite of the increasing of pollution by residue of synthetic pesticide in towns environment, this is an inevitable threat to citizen health, but the lack of mosquito management in private areas makes the level of infestation by *A. albopictus* out of control. According to the insect biology, one of the most important reproduction sites chosen by the insect are saucers flower pots and road drains, requiring a domestic and capillary use of the insecticide. Therefore, pesticide effects must be tested and performed in aqueous conditions.

Being biodegradable by action of sun light, neem products do not leave any residue on the field. The degradation of Az under field conditions is quite rapid and takes place by the effects of UV light, temperature, pH and microbial activity, avoiding accumulation. However, neem commercial success has been limited by the relatively high cost of the refined product and the low persistence of azadirachtin activity on crops exposed at sun light (Isman, 2006). These two aspects lead to explore the production of new neem products maintaining the insecticidal activity and helping the commercialization.

9. Analyzing neem cake products in four steps

The exact determination of neem products chemical composition can be considered the Mont Everest of the analytical study. Besides the aforementioned constituents of the neem oil, already object of several studies, like limonoids and fatty acids, determination of composition of neem cake composition is a hard, but necessary work. Apparently, this complexity could be of great importance for the larvicidal activity of neem cake, since minor compounds could be important copartecipants of the activity, being crucial in solubility and biodisponibility in accordance with the phytocomplex phylosophy. To face the complexity of neem cakes composition two analytical approaches were used: HPTLC was selected to evidence the total spread of constituents and HPLC to define quantities of limonoids.

High Performance Thin Layer Chromatography - HPTLC - is the last frontier of planar chromatography, becoming one of the best methods for control of quality, purity, stability and identity, in one word validation of complex botanical products (Reich, E. & Schibli, A. 2007). Besides the achieved great increasing in efficacy, due to the use of minor size silica gel that means larger surface, it is possible to perform high quality HPTLC analytical determinations, due to novel dedicated machinery, in order to finally achieve the necessary reliability, repeatability and flexibility. Substantially, the advantages of HPTLC are consistent with the rapidity and the possibility of analysing many samples at the same time under the same chromatographic conditions. The same HPTLC plate can be visualised with and without derivatisation using different light sources, obtaining an enormous quantity of information. Comparison is easy by the fingerprint approach. A fingerprint is the individual chromatographic track representing, as near as possible, the mixture of produced organic substances. By the fingerprint approach, it is possible to obtain a proper identification of the plant material, but also determine and assert the limits of the biological changes, without necessary identifying nor quantifying a specific compound(s). In HPTLC tracks of the same species variations are mainly quantitative, not qualitative. The fingerprint approach is very useful in the analysis of complex mixtures. HPTLC results a simple, rapid and useful method to obtain a general and almost complete information about the composition of neem cake products. Many products can be compared side-by-side with standards and

densitometry allows a quantitative analysis, opening planar chromatography to the 3D dimension. The fingerprint approach was introduced and accepted by WHO, as useful analytical technology for identification and quality validation of herbal products (WHO, 1991). The possibility of a correct use in estimation of AzA, AzB, salannin and nimbin in herbal extracts was demonstrated (Agrawal et al., 2005).
The study of neem cake products, based on determination of compositions and relative activities, was performed on four steps.

9.1 First step

HPTLC fingerprints of several neem cake marketed products on comparison with limonoids standards. Beside the expected differences between oil and deoiled, the HPTLC showed great variations in compositions. The Fig. 2 and 3 report a typical HPTLC fingerprint analysis on some of marketed neem products obtained by the collaboration of producers or importers; the plate is the same, but in Fig. 2 the tracks were visualized at the white light after derivatisation with anysaldehyde, whereas in Fig 3 the visualization at 366 nm was used, in order to obtain further information on fluorescent compounds. The plate starts with tracks 1-6 were same limonoid standards were reported. It is noteworthy that several standards appear not pure. This is an effect of the extreme sensibility of HPTLC. In fact, although the same standards appeared sufficiently pure at the NMR inspection, HPTLC is more inspective. Track 7 reports the total fingerprints of utilized limonoid standards. Track 8 is dedicated to the analysis of a sample of neem oil directly obtained from India. The fingerprint shows a presence of limonoids, but with salannin and nimbin as the most evident limonoids whereas AzA results a secondary components, instead of the most reported analytical data. It is also evident the presence of the oil components well evident as a strong spot near to the front of the plate. The HPTLC analyses of commercial neem cakes obtained from different producers, importers and markers revealed great differences in the compositions. As expected on the basis of deoiling process, great differences concerned the quantity of fatty compounds. These compounds can be evidenced as strong bands of red shining color at 366 nm, whose identity was confirmed by isolation by CC in *n*-hexane/ethylacetate 9:1 and identification by ^{1}H and ^{13}C NMR in $CDCl_3$.
Tracks 9-15 are dedicated to neem cakes, using methanol extracts to obtain the most complete extraction: in general, fingerprints are very similar, confirming positively the neem origin, as well as differences can be observed in the intensity or presence of single spots. Track 9 presents the methanol extract of the previous product of track 8. A second strong band can be now observed near the middle of the fingerprint, that was assigned to mixtures of alcohol and methylester derivatives of the former fatty acids by NMR analysis. It is evident that the presence and/or predominance of fatty constituents can have great influence in the physico-chemical properties, as well as activities, of the different neem cake products. The same spot is also evident in tracks 10 and 11, but not so present in the other fingerprints. The contents of limonoids also vary in each fingerprint, as better evidenced in Fig. 3. Focusing on the presence of salannin, its occurrence appears in all the products, although at glance the quantity can not be derived. Situation for nimbin and AzA is completely different.
Attention was focused on the product of track 14, containing limonoids, but also great quantity of fatty constituents. Therefore, we developed a method to clean the ethylacetate

extract from most of the fatty compounds first by extraction of the total extract with ethylacetate, followed by precipitation in ethylether/ethylacetate solution, obtaining as evident by the consequent fingerprint of track 16, two main results: a) differences in the presence of limonoids were better appreciated and solubility in polar solvent increased; b) it was evidenced the presence of a series of compounds strongly fluorescent at 366 nm light, previously obscured by the fatty band. Further studies are in progress to assign the definitive structures of these substances.

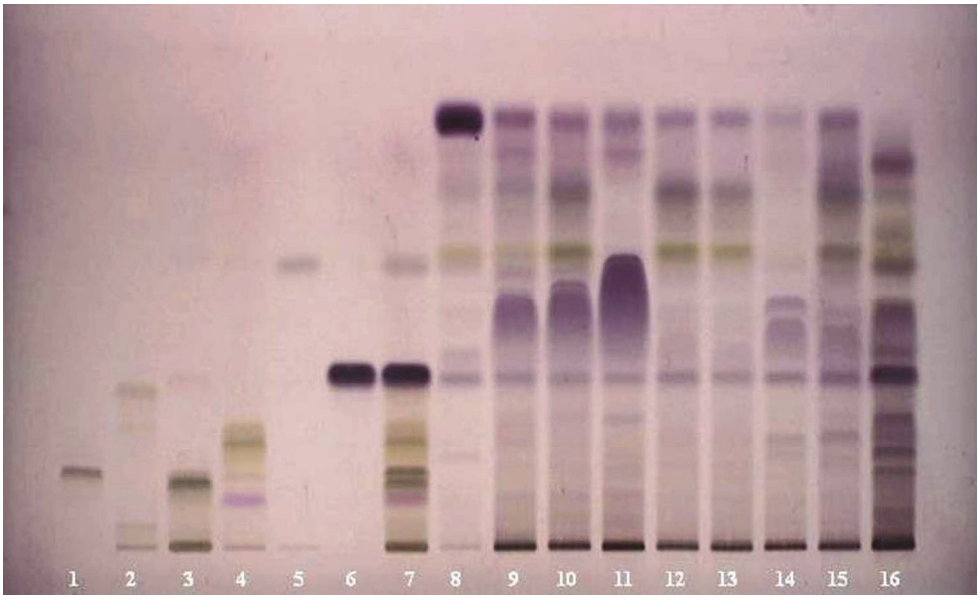


Fig. 2. HPTLC fingerprints of neem products and selected standards. Mobile phase: toluene, ethyl acetate (4:6). Derivatisation: Anisaldehyde. Visualization: white light. Tracks: **1** azadirachtin A, **2** azadirachtin B, **3** azadirachtin D (11-epi-azadirachtin A), **4** 11-deoxy-azadirachtin A, **5** nimbin, **6** salannin, **7** the previous standards all together, **8** neem oil marketed in India, **9** methanol extract of neem cake from India, **10-15** methanol extract of commercial samples of neem cakes; **16** neem cake of sample 14 re-extracted with ethyl acetate.

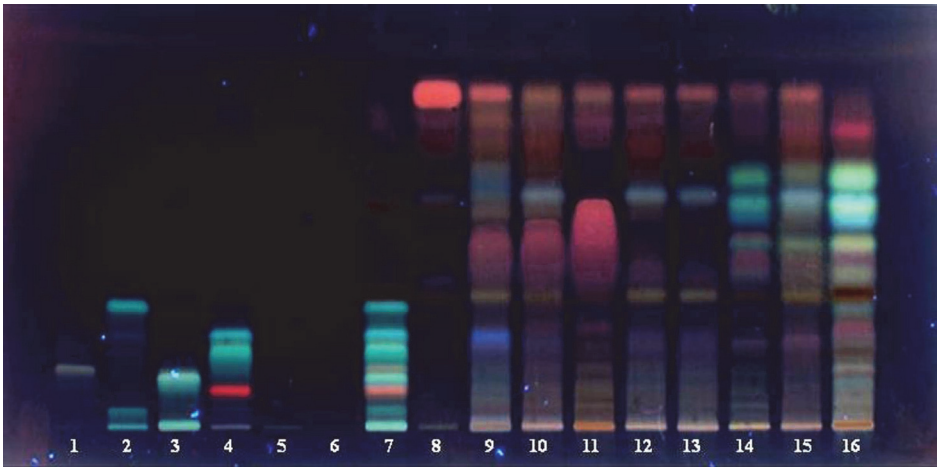


Fig. 3. The same tracks of Fig. 2 visualized at UV366 nm. In particular, by separation and NMR identification the main spots evident in the middle of the tracks 10-12 resulted as a mixture of fatty constituents.

9.2 Second step

Analysis of neem cake by HPLC. In order to better analyse the differences in neem cakes, neem cake of track 14, which was selected as test product for its complexity, was analyzed by HPLC for content in limonoids. Fig 4 shows the typical HPLC chromatogram of the methanol extract of neem cake, performed in gradient of water/CH_3CN 30-60% using a LC-18 column. The analysis, performed to evidence the nortriterpene presence, showed the following results: AzA (2750 ± 100 ppm), AzB (1000 ± 15 ppm), salannin (7980 ± 50 ppm), nimbin (1850 ± 100 ppm) (Nicoletti, 2010). Therefore, also after the industrial treatment of extraction, neem cake still contains relevant quantities of nortriterpenes.

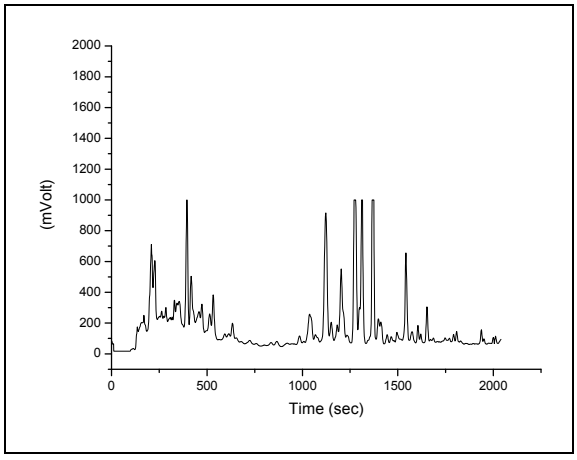


Fig. 3. Typical HPLC chromatogram of methanolic extracts of a neem cake products evidencing the limonoids in the middle of the chromatogram.

The main obtained evidences in composition analysis can be now summarized:

a. composition of neem cakes is different from that of neem oils.
b. differences in general composition of the marketed neem products were evident. The used repartition method resulted not efficient in separating the different constituents. The presence of limonoids in all extracts could be evidenced, although in different amounts as determined by HPLC. Thus, for instance, salannin resulted the most predominant limonoid, with low quantities of AzA. This result is in contrast with analysis of neem oil.
c. HPLC showed a precise figure of the limonoid contents, allowing a comparison between these data and larvicidal activities on *Aedes albopictus*.
d. constituents of different chemical structures from those already reported can be accumulated during processing steps.

9.3 Third step

Test of the larvicidal activity of different extracts. Therefore, the dried total extract was washed with *n*-hexane and then dissolved in a water/ethylacetate mixture, and the resulting water phase reextracted with *n*-butanol, in order to obtain four extracts partially concentrated in constituents of increasing polarity (named Hp for the *n*-hexane, Ep for the ethylacetate, Bp for the *n*-butanol and Wp for the water).

Larvicidal activities of the four extracts (Ep, Hp, Bp and Wp) of neem cake product of track 14 was determined, in order to locate presence and polarity of active principles. The test used *Aedes albopictus* a week old eggs, still laying on their paper substrate, after one day of drying, when submerged in the tested solutions, hatch. The results show that ovicidal activity doesn't occur when a week old eggs are deposited in neem derivatives. Tables 1, 2, 3 show that the percentage of hatching occurs without significant differences in all tested solutions in respect of the control.

The new born larvae were allowed to develop in the tested solutions and the larval mortality was assessed after 2-4-6-8-days. At day 8, Ep and Hp (Table 1) show a high larval mortality compared to control, to Bp and to Wp. In table 2 the Ep activity is compared to that of different concentration of AzA: at the highest AzA concentration, Ep present a higher mortality after 8 days. At day 8, the Ep (Table 3) shows the same effect, in terms of larval mortality, of the Dirachtin solutions at Az 10-100-1000 ppm.

Samples	Mean hatch (% ± DS)[1]	Mean mortality (% ± DS)[1] after 2 and 8 days	
		2	**8**
control (H_2O)	63.33 ± 21.86 [a]	6.90 ± 6.20 [a]	6.90± 6.20[a]
Bp	53.33 ± 5.77 [a]	6.67 ± 11.55 [a]	6.67 ± 11.55 [a]
Wp	43.33 ± 14.53 [a]	2.22 ± 3.85 [a]	15.56 ± 26.94 [a]
Ep	36.67 ± 14.53 [a]	4.95 ± 4.29 [a]	86.94 ± 5.17 [b]
Hp	35.56 ± 18.95 [a]	3.70 ± 6.42 [a]	88.78 ± 5.39 [b]

[1] Different letters in horizontal line indicate significant differences in hatching and mortality rate of larvae by Tukey's Test at 0,05 level.

Table 1. Comparison of the activity of various neem-cake extracts against *Aedes albopictus* as indicated by the hatching rates of eggs and larval mortality after 2 and 8 days (Tukey's Test).

Samples	Mean hatch (% ± DS)[1]	Mean mortality (% ± DS) [1] after 2 and 8 days	
		2	**8**
control (H_2O)	63.33 ± 21.86 [a]	6.90 ± 6.20 [a]	6.90 ± 6.20[a]
Az_A 0.1 ppm	44.44 ± 1.92 [a]	2.56 ± 4.44 [a]	10.26 ± 17.76 [a]
Az_A 0.5 ppm	55.33 ± 14.53 [a]	3.17 ± 5.50 [a]	3.17 ± 5.50 [a]
Az_A 1.0 ppm	47.78 ± 15.75 [a]	0,0 ± 0,0 [a]	5.79 ± 5.57 [a]
Az_A 5.0 ppm	54.44 ± 5.09 [a]	0.0 ± 0.0 [a]	27.69 ± 18.22 [a]
Az_A 10.0 ppm	48.89 ± 6.94 [a]	13.21± 5.06 [b]	80,49 ± 10.99 [b]
Ep	36.67 ± 14.53 [a]	4.95 ± 4.29 [a]	86.94 ± 5.17 [b]

[1] Different letters in horizontal line indicate significant differences in hatching and mortality rate of larvae by Tukey's Test at 0.05 level.

Table 2. Comparison of the activity of Ep and technical azadiracthin solutions at various Az_A concentrations against *Aedes albopictus* as indicated by the hatching rates of eggs and larval mortality after 2 and 8 days. (Tukey's Test)

Samples	Mean hatch (%±DS) [1]	Mean mortality(%±DS) [1] after 2 and 8 days	
		2	**8**
control (H_2O)	28.89 ± 5.09 [a]	3.70 ± 6.42 [a]	14.44 ± 17.11 [a]
Dirachtin(Az 1.0ppm)	24.44 ± 5.09 [a]	31.75 ± 2.75[a]	36.51 ± 5.50 [a]
Dirachtin(Az 10.0ppm)	31.11 ± 1.92 [a]	22.22 ± 22.22 [a]	85.56 ± 6.76 [b]
Dirachtin(Az 100ppm)	27.78 ± 1.92 [a]	51.85 ± 37.64 [a,b]	92.13 ± 6.85 [b]
Dirachtin(Az 1000ppm)	30.0 ± 3.33 [a]	100.0 ± 0.0 [b]	100.0 ± 0.0 [b]
Ep	30.42 ± 12.73 [a]	3.75 ± 3.89 [a]	84.34 ± 4.65 [b]

[1])Different letters in horizontal line indicate significant differences in mortality rate of larvae by Tukey's Test at 0.05 level.

Table 3. Comparison of the activity of Ep and Az commercial formulation at various concentrations against *Aedes albopictus* as indicated by the hatching rates of eggs and larval mortality after 2 and 8 days. (Tukey's Test)

9.4 Forth step

Evaluation of larvicidal activities of neem cakes acqueous solution, obtained by soaking 3,7 g in 100ml of raining water, in relation with limonoids contents. The previous studies can be now combined to obtain a final result. The HPTLC analysis of the six selected marketed neem products was performed to properly evidence differences in compositions (Fig. 4).

Fig. 4. HPTLC analysis of selected neem cakes. Tracks 1-6 fingerprints of six marketed neem cakes; tracks 7-8 neem cake of track 6 reextracted with ethylacetate and defatted in two concentrations; track 9 nimbin; track 10 salannin.

HPLC analysis confirmed the prevalence of salannin, whose quantity changes greatly in the different products. The larvicidal effects evidence a good correspondence between salannin content and larvicidal activity.

Neem cake	AzA (ppm)	Nimbin	Salannin
1	79	26	858
2	15	84	266
3	107	117	190
4	185	126	1260
5	184	321	2700
6	40	250	1390

Table 4. HPLC results on quantitative determination of main limonoids in neem cake marketed products.

Neem cake	2 days	3 days	4 days
1	2.2 ± 3.8	94.1 ± 10.2	97.9 ± 3.6
2	50 ± 50	50 ± 50	70.0 ± 30
3	3.0 ± 5.2	3.0 ± 5.2	3.0 ± 5.2
4	93.3 ± 5.9	93.3 ± 5.9	93.3 ± 5.9
5	86.7 ± 23.1	100.0 ± 0.0	100 ± 0
6	65.3 ± 17.7	90.2 ± 9.2	100 ± 0
Control	22.6 ± 20.9	22.6 ± 20.9	22.6 ± 20.9

Table 5. Larvicidal effects of different neem cakes on *A. aldopictus*

10. Concluding

The complexity of neem cake requires a multidevice approach, in order to obtain a great quantity of data in accordance with the different types of natural products present. Total information derives from the complementary use: HPTLC for general composition, NMR for structural determination, HPLC for quantitative determination.
The activity observed is mainly preliminary and must be confirmed with the study on the physiological effects on larvae. A comparison of the results reported with the outcome from other studies concerning the neem products is not easy. Differences can be attribute to the origin of the products, concentrations of active ingredients, the target mosquito, modes of application. However, larvicidal activity of neem cakes on Asiatic Tiger was evidenced. Activity is in some way related to salannin and/or limonoids contents, but the co-operative influence of other constituents must be considered.
The enormous quantity of different compounds allows the possibility of increase the presence of selected constituents by chemical treatment as evidenced in the fingerprint of track 16.
Future steps in the validation of neem cake as possible raw material for the development of a new domestic insecticide will be the study of the physiological effects on larvae, the exact determination of the composition of the most active neem cake products and the development of a solution containing most of the active products and to be used in aqueous environment, like puddles, small marshes, saucers, gutters.
In 1992 U.S. Academy of Science published a report entitled prophetically "Neem, a tree for solving global problem".

11. Acknowledgements

Thanks to the producers and distributors for providing the neem cake products used. A special thanks to Dr. Maurizio Calvitti and Riccardo Moretti UTAGRI Technical Unit of the ENEA-CR CASACCIA who provided the eggs and larvae for all tests.

12. References

Agrawal H., Kaul N., Paradkor A.R., Mahadite A. 2005 Standardization of Crude Extracts of neem kernels (*Azadirachta indica* A. juss) and commercial neem based formulations using HPTLC and extendel Length Packed-Column SFC Methods *Chromatographia*, 62, 180-195.

Amorose T. 1995. Larvicidal efficacy of neem (Azadirachta indica) oil and defatted cake on *Culex quinquefasciatus* say. *Geobios*, 22, 169–173.

Boeke S.J., Boersma M.G., Alink G.M., van Loon J.J., van Hnis A., Dicke M., Rietjens I.M. 2004 Safety evaluation of neem (*Azadirachta indica*) derived pesticides Journal of Ethnopharmacology 94, 25-41.

Biswas K., Chattopadhayay J., Banerjee R.M., Bandyopdyodhyay U. 2002 Biological activities and medical properties of neem (*Azadirachta indica*) *Current Science*, 82, 1336-1345.

Gajalakshimi S. and Abbasi S.A. 2004 Neem leaves as a source of fertilizer-cum-pesticide vermicompost *Biosource Technology*, 92, 291-296.

Gandhi M., Lal R., Sarkaranarayaman A., Baneyel C.K., Sharna P.L. 1988 Acute Toxicity of the oil from *Azadirachta indica* seed (neem oil) *Journal of Ethnopharmacology*, 23, 39-51.

Gopal M., Gupta A., Arunachalam V., Maga S.P. 2007 Impact of azadirachtin, an insecticidal allelochemical from neem on soil microflora, enzyme and respiratory activities *Bioresource Technology*, 98, 3154-3158.

Gratz N.G. 2004 Critical review of the vector status of *Aedesalbopictus Medical Veterinary Entomology*, 18, 215–227.

Howard A.F.V., Adongo E.A., Hassanali A., Omlin A.X., Wanjoys A., Zhou G., Vulule J. 2009 Laboratory Evaluation of the Aqueous Extract of *Azadirachta indica* (Neem) Wood Chippings on *Anopheles gambiae* s.s. (Diptera; Culicidae) *Mosquitoes Entomological Society of America*, 46, 107-114.

Johnson S., Morgan E.D. 1997 Supercritical fluid extraction of oil and triterpenoids from neem seeds *Phytochemical Analysis*, 8, 228-232.

Kurose K. and Yatagai M. 2005 Components of the essential oils of *Azadirachta indica* A. Juss, *Azadirachta siamensis* Velton and *Azadirachta excelsa* (Jack) Jacobs and their comparison *Journal of Wood Science*, 51, 183-188.

Mongkholkhajornsilp D., Supaporn D., Douglas P.L., Elkamel A., Teppaitoon W., Pongamphai S. 2005 *Journal of Food Engineering*, 71, 331-340.

Mulla M.S. and Su T. 1999 Activity and biological effects of neem products against arthropods of medicinal and veterinary importance *Journal of American Mosquitos Control Association*, 15, 133-152.

Naqvi S.N., Ahmed S.O., Mohamed F.A. 1991 Toxicity and IgR (insect regularity effect) of two neem products against *Aedes aegypti* (PCSIR strain) *Pakistan Journal of Pharmaceutical Science*, 4, 71–76.

Ndione R.D., Faye O., Ndiaye M., Afoutou J.M. 2007 Toxic effects of neem products (*Azadirachta indica* A. Juss) on *Aedes aegypti* Linnaeus 1763 larvae *African Journal of Biotechnology*, 6, 2846-2854.

Nicoletti M., Serafini M., Aliboni A., D'Andrea A., Mariani S. 2010 Toxic effects of neem cake extracts on *Aedes albopictus* larvae *Parasitology Research*, 107, 89-84.

Okumu F.O., Knols B.G.J., Fillinger U. 2007 Larvicidal effects of a neem (*Azadirachta indica*) oil formulation on the malaria vector *Anopheles gambiae Malaria Journal*, 6, 63-67.

Punt L. 2000 Neem based insecticides Homes & Gardens News. July/August, 6.

Rao D.R., Reuben R., Venugopel M.S., Nagesampagi B.A. , Schumetterer 1992 Evaluation of neem, *Azadirachta indica*, with and without water management for the control of culicine mosquito larvae in rice-field Medical Veterinary Entomology, 6, 315-324.

Rao V.K, Kowale B.N., Verna A.K. 2003 Effect of feeling washed neem (*Azadirarachta indica*) seed kernel cake on the quality, lipid profile and fatty acid composition of goat meat *Small Ruminant Research*, 47, 213-219.

Reich, E.; Schibli, A. 2007 High-performance thin-layer chromatography for analysis of medicinal plants. Thieme Medical Publishers Inc.: New York.

Silva J.C.T., Gulab N.J., D'are R., Oliveira L., Brown L. 2007 Purification of seven tetraterpenoids in neem (*Azadirachta indica*) seed by counter-current chromatography sequentially followed by isocratic preparative reversed-phase high-performance liquid chromatography *Journal of Chromatography A*, 1151, 203-210.

Su T. and Mulla A.S. 1999 Oviposition bioassay responses of *Culex tarsalis* and *Culex quinquefasciatus* to neem products containing azadirachtin *Entomologia Experimentalis et Applicata*, 91, 337-345.

Wandscheer C.B., Duque J.E., da Silva M.A.N., Fukuyama Y., Wohlke J.L., Adelmann J., Fontana J.D. 2004 Larvicidal action of ethanolic extracts from fruit endocarps of *Melia azedarach* and *Azadirachta indica* against the dengue mosquito *Aedes aegypti*. *Toxicon*, 8, 829–835.

World Health Organisation 1991 Guidelines for the Assessment of Herbal Medicine, Munich. World Health Organisation, Geneva, Switzerland.

World Health Organisation 2004 Global strategic framework for integrated vector management. World Health Organisation, Geneva, Switzerland.

World Health Organisation 2005 Guidelines for laboratory and field testing of mosquito larvicides. World Health Organisation, Geneva, Switzerland.

Varie P.S. 1996 Indian Medicinal Plants. A compendium of 500 species. Vaidyaratnam. Arya Vaidya Sala Kottakal. Orient Longman.

Vazeille M., Jeannin C., Martin E., Schaffner F., Failloux A.B. 2008 Chikungunya: a risk for Mediterranean countries? *Acta Tropical*, 105, 200–202.

Verna A.K., Sastry V.R.B., Agrawal D.K. 1994 Feeding of water neem (*Azadirarachta indica*) seed kernel cake to growing goats *Small Ruminant Research*, 15, 105-111.

Zebitz C.P.W. 1987 Potential of neem extracts in mosquito control. In: Schmutteret H. & Ascer K.R.S. (eds) Natural Pesticides from the neem tree and other tropical plants. Proc. 3rd Int. Neem Cong. Nairobi 1986 GTZ Eschborn, 555-573.

19

Reproductive and Developmental Toxicity of Insecticides

Ferdinand Ngoula[1], Omer Bébé Ngouateu[2], Jean Raphaël Kana[1],
Henry Fualefac Defang[1], Pierre Watcho[3],
Pierre Kamtchouing[2] and Joseph Tchoumboué[1]
[1]*Department of Animal Sciences, Faculty of Agronomy and Agricultural Sciences, University of Dschang*
[2]*Department of Animal Biology and Physiology, Faculty of Sciences, University of Yaoundé I*
[3]*Department of Animal Biology, Faculty of Sciences, University of Dschang*
Cameroon

1. Introduction

Under the pretext of demographic growth with all its consequences, agricultural production resorts to the use of a varied and a large quantity of insecticides to improve the production and preservation of foodstuffs. Thus, the use of insecticides has increased rapidly and is now widespread to the lowest level of agricultural production.

Insecticides are products of chemical or biological origin that control insects (Ware and Whitacre, 2004). They include ovicides and larvicides used against the eggs and larvae of insects respectively and are used in agriculture, medicine, industry and the household. Insecticides are believed to be the major factors behind the increase in agricultural productivity in the 20th century (van Emden and Pealall, 1996). Control insects may result from killing the insect or otherwise preventing it from engaging in behaviors deemed destructive. Insecticides may be natural or manmade and are applied to target pests in a myriad of formulations and delivery systems (sprays, baits, slow-release diffusion, etc.). Biotechnology has, in recent years, even incorporated bacterial genes coding for insecticidal proteins into various crops to kill pests that feed on them (Ware and Whitacre, 2004). Obviously, this abundant and diversified use of insecticides constitutes a danger not only for aquatic and terrestrial biodiversity, but also for humans because of their presence in food chains.

The World Health Organization (WHO) estimates at 20,000 the number of deaths caused by pesticides each year worldwide with a substantial proportion due to insecticides (Darren et al., 2003). These incidents are particularly common in developing countries; where the marketing of pesticides do not respect international quality standards. Moreover, many studies conducted all over the world report undeniable links between insecticides and serious health consequences including endocrine disruption and fertility problems (Colborn et al., 1993; Colborn et al. 1996, Andersen et al., 2000), cancers (Ben Rouma et al. 2001;

Cabello et al., 2001, Clark et al., 2002; Darren et al., 2003), depression of the immune system, genotoxicity, aplasic anemia. (Pesticide Action Network Belgium, 1999)
Exposure to insecticide has been associated in animals and humans with occurrence of spontaneous abortion, low birth weight, birth defects, change in male: female sex ratio of offspring, inhibition of spermatogenesis and ovogenesis, destruction of seminiferous epithelium, hydrocels resulting to reduction in fertility (Ngoula et al., 2007[a]; Ngoula et al., 2007[b]; Farag et al., 2000; Farag et al., 2010; Shalaby et al., 2010; Chung et al., 2002; Moline et al., 2000; Sobrazo and Bustos-Obregòn, 2000[a]; Delemarre-van de Waal, 1993; Villeneuve, 1972; Vartiainen et al., 1999; Lenselink et al., 1993; Talens and Wooley, 1973; Vogin et al., 1971).
Of Hundreds of insecticides available in the market, few were studied for their impact on reproduction and development. On the other hand, information related to this domain is not only scanty, but also very scattered. The objective of this chapter is to review the reproductive and developmental toxicity of insecticides after an overview of animal reproduction and development. Finally, recommendations for insecticides users and researchers will be proposed.

2. Animal reproduction and development

Reproduction can be defined as the process by which an organism continues its species. In simple terms, it is the process by which organisms create descendants (Wikibooks, 2007).

2.1 Male reproductive system

The reproductive role of the male is to produce and deliver sperm to impregnate the female. To carry out these functions, a male has internal and external sexual organs. These structures include the testes, several tubules that carry sperm out of the testes, various glands, and the penis. In most mammalian species, including human, the male's external reproductive organs are the scrotum and penis. The internal reproductive organs consist of gonads that produce gametes (sperm cells) and hormones, accessory glands that secrete products essential to sperm movement, and ducts that carry out the sperm and glandular secretions (Campbell and Reece, 2005). Inside the testis is a network of fine-diameter tubes called seminiferous tubules. Sertoli cells, nourish, support, and protect developing germ cells, which undergo cell division by meiosis to form spermatozoa (immature sperm). Prostate secretions are rich in zinc, citric acid, antibiotic like molecules, and enzymes important for sperm function. During sexual excitation, the bulbourethral glands produce a droplet of alkaline fluid that neutralizes residual urine in the urethra, protecting the sperm from its acidity (Robinson, 2001). Table 1 summarizes the function of the male reproductive system.

2.2 Female reproductive system

The primary function of the female reproductive system is to produce gametes, the specialized cells that contribute half of the total genetic material of a new individual. The female reproductive system has several additional functions: to be the location for fertilization, to protect and nourish the new individual during the gestation period, and to nourish the newborn postpartum, through lactation and nursing (Weck, 2002).
The female's external reproductive structures include: the clitoris and two sets of labia which surround the clitoris and vaginal opening. The internal organs are a pair of gonads

ORGANS	FUNCTION
Testis with seminiferous tubules	Sperm and testosterone production
Collecting ducts	Transport and storage
Epididymis	Transport, maturation and ejaculation
Vas deferens	Transport and ejaculation
Seminal vesicles	Secretion of thick liquid to transport sperm
Prostate gland	Secretion of alkaline solution to neutralize the urine and female system
Cowper's gland	Secretions may lubricate, flush out urine or form a gelatinous plug
Urethra	Passage for urine and sperm
Penis	Copulation

Table 1. Function of male reproductive system.

(ovaries) and a system of ducts and chambers that carry gametes and house the embryo and fetus (Campbell and Reece, 2005). The functions of the female reproductive organs are summarized in Table 2.

ORGANS	FUNCTION
Ovaries	Production of germ cells and sex hormones
Ducts	Sperm migration, site of fertilization, transport of the fertilized ovum to the uterus
Uterus	Site of fixation, development and growth of the conceptus
Vulva	Copulation (the vagina receives the semen from male penis)
Bartholin Glands	Secretions may lubricate
Mammary glands	Feeding of the newborn

Table 2. Functions of female reproductive system.

2.3 Regulation of the reproductive system

In females, the secretion of hormones and the reproductive events they regulate are cyclic. Whereas males produce sperm continuously, females release only one egg or a few eggs at a specific time during each cycle (Campbell and Reece, 2005).

The secretory and gametogenic functions of the gonads are both dependent on the secretion of the anterior pituitary gonadotropins, FSH, and luteinizing hormone (LH) (Figure 1). The sex hormones and inhibin B feedback to inhibit gonadotropin secretion. In males, gonadotropin secretion is noncyclic; but in postpubertal females an orderly, sequential secretion of gonadotropins is necessary for the occurrence of menstruation, pregnancy, and lactation (Barrett et al., 2010).

Sperm production and androgen synthesis are controlled by a complex feedback loop involving the testes, hypothalamus, and pituitary gland. The pituitary controls the function of the testis by producing follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH stimulates spermatogenesis, in part by affecting Sertoli cells, while LH stimulates androgen production by interstitial cells. Pituitary production of these hormones depends on secretion of gonadotropin-releasing hormone (GnRH) by the hypothalamus which can be

stimulated by the cerebral cortex. Elevated levels of GnRH initiate puberty. The production of LH is controlled by the actions of testosterone on the hypothalamus and pituitary. The testis can control brain function. If testosterone concentration is elevated, this hormone inhibits production of GnRH by the hypothalamus; subsequently, LH and FSH production decreases (Palladino, 2002). Hormones also coordinate functions in several different organs at the same time. Considerable coordination among the organs of the female reproductive tract is required. Reproduction will not be successful unless ovulation at the ovary occurs near the time when the uterus is prepared to receive the pre-embryo and, soon thereafter, begin forming the placenta.

Without a functional placenta the pregnancy will not continue very long after implantation of the blastocyst. Surrounding the tubules are clusters of interstitial cells, which synthesize testosterone secretion into the bloodstream. Testosterone is present in infant boys, although synthesis increases dramatically at puberty around the age thirteen. This increase stimulates the onset of spermatogenesis and development of accessory sex glands. All male reproductive organs require testosterone for functions such as protein synthesis, fluid secretion, cell growth, and cell division. Androgens also play an important role in the male sexual response and stimulate secondary sex characteristics such as skeletal development, facial hair growth, deepening of the voice, increased metabolism, and enlargement of the testes, scrotum, and penis.

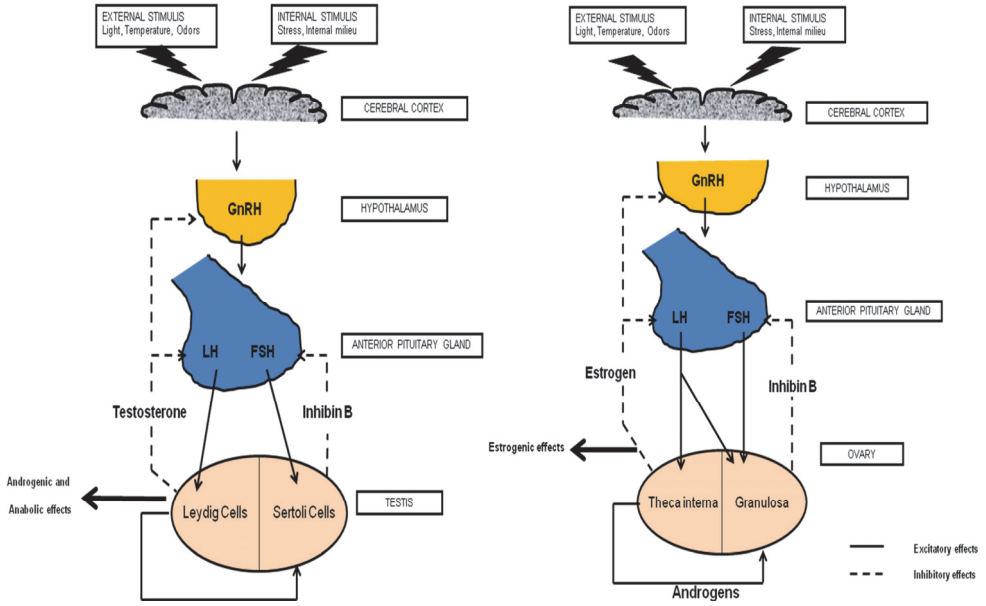


Fig. 1. Postulated mechanisms of regulation of male (on the left) and female (on the right) reproductive function (Barrett et al., 2010 modified).

2.4 fertilization, growth and development of conceptus

Fertilization occurs when sperm and oocyte cell membranes fuse. Once in the female reproductive tract, prostaglandins in the semen cause thinning of the mucus at the opening of the uterus and stimulate contractions of the uterine muscles, which help move the semen up the uterus. The alkalinity of the semen helps neutralize the acidic environment of the vagina, protecting the sperm and increasing their motility. When ejaculation takes place, the semen coagulates, making it easier for uterine contractions; then anticoagulants liquefy the semen, and the sperm begin swimming through the female tract (Campbell and Reece, 2005). Following coitus, exposure of sperm to the environment of the female reproductive tract causes capacitation, removal of surface glycoproteins and cholesterol from the sperm membrane, enabling fertilization to occur. Fusing of the first sperm initiates the zona reaction. Release of cortical granules from the acrosome causes biochemical changes in the zona pellucida and oocyte membrane that prevent polyspermy (Klein and Enders, 2007). Development begins with fertilization, the process by which the male gamete (sperm), and the female gamete (oocyte), unite to give rise to a zygote (Sadler, 2006). Fertilization of an egg by a sperm also called conception in Human occurs in the oviduct about 24 hours later, the resulting zygote begins dividing, a process called cleavage. Cleavage continues, with the embryo becoming a ball of cells by the time it reaches the uterus 3 to 4 days after fertilization. By about 1 week after fertilization, cleavage has produced an embryonic stage called the blastocyst, a sphere of cells containing a cavity. In a process that takes several more days for completion, the blastocyst implants into the endometrium. The embryo secretes hormones that signal its presence and control the mother's reproductive system. One embryonic hormone, human chorionic gonadotropin (HCG), acts like pituitary LH to maintain secretion of progesterone and estrogens by the corpus luteum through the first months of pregnancy in the absence of this hormonal override, the decline in maternal LH due to inhibition of the pituitary would result in menstruation and loss of the embryo (Campbell and Reece, 2005). In human, growth in length is particularly striking during the third, fourth, and fifth months, while an increase in weight is most striking during the last 2 months of gestation. In Human, the length of pregnancy is considered to be 280 days, or 40 weeks after the onset of the last normal menstrual period (LNMP) or, more accurately, 266 days or 38 weeks after fertilization. There are high risks of malformation during the embryogenesis. Birth defect, congenital malformation, and congenital anomaly are synonymous terms used to describe structural, behavioral, functional, and metabolic disorders present at birth. Terms used to describe the study of these disorders are teratology and dysmorphology (Sadler, 2006).

3. Insecticides

3.1 Definition and classification

Insecticides are a group of substance belonging to pesticides. Pesticides previously known as agricultural chemicals are economic poisons that are used to control, kill or repel pest. Depending on the target pest, pesticide can be subclassified into a number of categories namely algicide, fungicide, herbicide, nematocide, mollucide, insecticide, acaricide, rodenticide etc. Depending on the toxicity, formulation concentration, and the pattern use, pesticides can be classified as “general” or “restricted” used. The United States Environmental Protection Agency (US EPA) has developed “category use” definitions based on toxicity. Thus Category I pesticides are highly hazardous, classified as restricted use and

have an oral LD_{50} less than or equal to 1.0mg/kg of body weight; category II are moderately toxic pesticides with an oral LD_{50} less or equal to 500mg/kg; category III are generally non toxic pesticides and have an oral LD_{50} less or equal to 15 000 mg/kg The primary classes of pesticides in use today are fumigants, fungicides, herbicides and insecticides (Hodgson, 2004).

Insecticides can be divided into:

Organochlorines which are insecticides that contain carbon, hydrogen and chlorine. They are also known as chlorinated hydrocarbons, chlorinated organics, chlorinated insecticides, or chlorinated synthetics. Today this group is scarcely used.

Organophosphates (OPs) is the generic term that includes all insecticides containing phosphorus. All Ops are esters of phosphorus having varying combination of oxygen, carbon, sulfur and nitrogen attached.

Organosulfurs contain two phenyl rings with sulfur as the central atom (instead of carbon like in DDT).With very low toxicity to insects, they are used only as acaricides (miticides).

Carbamates are insecticides derivatives of carbamic acid. They inhibit cholinesterase as OPs do.

Formamidines comprise a small group of insecticide used to control OP-and carbamate-resistant pests.

Dinitrophenols have a broad range of toxicity as herbicides, insecticides, ovicides, and fungicides.

Organotins is mainly used as an acaride.

Pyrethroids are very stable in sunlight and are generally effective against most agricultural insect pest when used at the very low rates.

Nicotinoids are new class of insecticides with a new mode of action.

Spinosyns are represented by spinosad which is a fermentation metabolite of the actinomycete *Saccharopolyspora spinosa.*

Fiproles (or Phenylpyrazoles) are used for the control of many soil and foliar insects.

Pyrroles are used as insecticide-miticide on cotton and experimentally on corn, soybeans, vegetables, tree and vine crops etc.

Pyrazoles consist of tebufenpyrad and fenpyroximate which are miticides with limited effectiveness on psylla, aphids, whitefly, and thrips.

Pyridazinones has only Pyridaben as a member of this class. It is a selective insecticide and miticide, aloso effective against thrips, aphids, whiteflies and leafhorpprs.

Quinazolines offer a unique chemical configuration, consisting only of one insecticide, fenazaquin which is a contact and stomach miticide.

Benzoylureas are a group of insecticides that act as insect growth regulators. Their greatest value is in the control of caterpillars and beetles larvae.

Botanicals are natural insecticides, toxicants derived from plants.

Synergist (or Activators) are not themselves considered toxic or insecticidal, but are materials used with insecticides to synergize or enhance the activity of insecticides.

Antibiotics comprise *avermectins* which are insecticidal, acaricidal.

Fumigants are small, volatile, organic molecules that become gases at 40°F. They are generally heavier than air and commonly contain one or more of the halogens (Br, Cl or F).

Insect repellents include smoke, plants hung in dwelling or rubbed on the skin as the fresh plant or its brews, oils, pitches, tars, and varied earths applied to the body (Ware, 2001).

4. Reproductive toxicity of insecticides

4.1 Effects of insecticides on male reproductive system

Insecticides can affect the male reproductive system at one of several sites or at multiple sites. These sites include testes, the accessory sex glands, and the central nervous system, including the neuroendocrine system (Moline et al., 2000). Insecticides may directly damage spermatozoa, alter Sertoli cell or Leydig cell function, or disrupt the endocrine function in any stage of hormonal regulation (hormone synthesis, release, storage, transport, and clearance; receptor recognition and binding; thyroid function; and the central nervous system). These mechanisms are described with respect to the effects of insecticides exposure in vitro and in vivo (Mathur et al., 2010).

4.1.1 Effects of carbamate insecticides on male reproductive system

Subchronic administration of Methomyl, a Carbamate insecticide, to male rat significantly decreased the fertility index, weight of testes and accessory male sexual glands, serum testosterone level and sperm motility and count, but increased sperm cell anomalies. It induced testicular lesions characterized by moderate to severe degenerative changes of seminiferous tubules and incomplete arrest of spermatogenesis. These toxic effects are not persistent (Shalaby et al., 2010). Propoxur (2-isopropoxy-phenyl-N-methylcarbamate), a carbamate pesticide, administered to adult male Wistar rats for 90 successive days led to a concentration-dependent increase in relative weights of testis and epididymis and a decrease in sperm density, serum and intratesticular total cholesterol concentrations, and intratesticular total proteins in treated rats. Propoxur had no significant effect on gestation, fertility and parturition indices, average birth weight, litter size and pups sex ratio of untreated female rats mated with treated males rats (Ngoula et al., 2007[a]).

Two studies at a carbaryl manufacturing factory have shown that carbaryl exposure affects the quantity and quality of sperm produced by the workers. A second study of the same sperm samples found that the number of sperm anomalies was increased in workers who were being exposed to carbaryl (Wyrobeck et al., 1981). Rani et al. (2007) evaluated the carbaryl exposure and showed distorted shape of seminiferous tubules, disturbed spermatogenesis, and accumulation of cellular mass in the lumen of tubules, oedema of the interstitial spaces and loss of sperms of varying degrees in testes.

Studies on laboratory animals in addition to limited human data showed an association between carbaryl exposure and decreased semen quality.

4.1.2 Effects of organochlorine insecticides on male reproductive system

The chlorinated hydrocarbon insecticides were introduced in the 1940s and 1950s and include familiar insecticides such as DDT, methoxychlor, chlordane, heptachlor, aldrin, dieldrin, endrin, toxaphene, mirex, and lindane. The chlorinated hydrocarbons are neurotoxicants and cause acute effects in the transmission of nerve impulses.

Detectable levels of lindane, DDT, and dieldrin were found in German men, with the highest levels in chemistry students (Alegakis et al., 1996). DDE, aldrin, endosulfan, and isomers of hexachlorocyclohexane (HCH), were detected in men in India (Potashnik et al., 1987).

Exposure to persistent organoclorine pollutants has been associated with human perturbations of the sperm X:Y chromosome ratio (Niederberger, 2005). On the other hand, a high dose of 2-bromopropane decreases spermatogenesis by adversely affecting

spermatogonia followed by depletion of spermatocytes, spermatids, and spermatozoa, with subsequent testicular atrophy (Hwa–Young et al., 1999). Methoxychlor induces oxidative stress in the epididymis and epididymal sperm by decreasing antioxidant enzymes, possibly by inducing reactive oxygen species (Latchoumycandane et al., 2003).

Pant et al. (1995) reported a dose dependent decrease of the weight of epididymides, seminal vesicles, ventral prostate and coagulating glands in male rats exposed to Carbofuran (0.1, 0.2, 0.4 or 0.8 mg kg-1 body weight, 5 days/week for 60 days). Decreased sperm motility, reduced epididymal sperm count along with increased morphological abnormalities in head, neck and tail regions of spermatozoa were observed in rats exposed to 0.2, 0.4, or 0.8 mg carbofuran kg-1 body weight. Histologically, the results indicated the toxicity of carbofuran on testes depending on the doses. The changes predominantly consisted of moderate oedema, congestion, damage to Sertoli cells and germ cells, along with the accumulation of cellular debris and presence of giant cells in the lumen of a few seminiferous tubules which showed disturbed spermatogenesis with the higher doses of carbofuran.

A recent study suggests that endosulfan exposure may delay sexual maturity and interfere with hormone synthesis in male children (Narayana et al., 2004). Jaiswal et al. (2005) reported pre-treatment with 5-aminosalicylic acid (5-ASA) significantly reduced sperm-shape abnormalities in endosulfan-treated rats. The number of abnormal sperm in the epididymis was markedly increased by endosulfan treatment. Histopathological analysis of seminiferous tubules and Leydig cells showed significant protection from endosulfan-induced tissue damage such as necrosis. The population of Sertoli cells increased and the lumen of the seminiferous tubules contained a greater number of spermatids. There was a corresponding increase in the number of Leydig cells. Rao et al. (2005) investigated the effect of L-ascorbic acid on postnatal exposure of endosulfan induced testis damage in the rat. Endosulfan affected the testicular function enhancing the incidence of abnormal spermatozoa, decreasing the sperm count and sperm motility.

In a study carried out on the reproductive functions of 32 sprayers men exposed to 2,4-D, and after four days of sexual inactivity the results of their sperm analysis, compared with unexposed workers, showed a significantly high levels of asthenospermia, necrospermia and teratospermia (Lerda and Rizzi, 1991). An increase in the germ cells and sperm head abnormalities was observed after oral administration of 2,4-D at 3.3 mg kg(-1) in male rats for three and five consecutive days (Amer and Aly, 2001).

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) induces oxidative stress in the epididymis and epididymal sperm by decreasing the antioxidant enzymes through induction of reactive oxygen species. Male rats exposed to TCDD display reduced fertility, delayed puberty and altered reproductive organ weights (Bell et al., 2007). TCDD- exposed male rats displayed decreased numbers of sperm and increased numbers of abnormal sperm in the epididymis (Faqi et al., 1997).

HCH exposure (50 mg or 100 mg kg-1 body weight day-1, 5 days in a week for 120 days) also led to a decrease in epididymal sperm count, sperm motility and an increase in the percentage of abnormal sperm (Prasad et al., 1995).

Lindane, an organochlorine pesticide, impairs testicular functions and fertility. Lindane has direct action on reproduction and also carcinogenic properties. Treatment with 1-40 mg of lindane/kg body weight disrupted testicular morphology, decreased spermatogenesis and impaired reproductive performances in males (Page et al., 2002).

The weights of the testis, epididymis, seminal vesicles and ventral prostate decreased in methoxychlor treated rats (Latchoumycandane and Mathur 2002). The activities of antioxidant enzymes such as superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase decreased in testes. The levels of hydrogen peroxide generation (H2O2) and lipid peroxidation increased in testis of the rats treated with methoxychlor.
According to Waissmann (2003); Hakin and Oates (1997), DDT and some organic solvents lead to decreased fertility and altered sperm counts DDT can also delay puberty (Santamarta, 2001; Jequier, 2002; Waissmann, 2003; Metzler, 2002; Moreira and Wolff 2003).
Dioxins can affect libido and fertility, causing changes in the sexual behavior of male fish, birds, mammals, and reptiles as reported by Assunção and Pesquero (1999), Ribeiro (2003), Giwercman et al. (1993). Tetrachloro-dibenzo-p-dioxin (TCDD) can interfere with libido (Hakin and Oates, 1997). The effects of high exposure to TCDD and "TCDD-like" compounds on important sites for development and reproduction have been also been recognized by Eskenazi and Kimmel (1995).
Endosulfan exposure in male children may delay sexual maturity and interfere with sex hormone synthesis (Saiyed et al., 2003).
Ben et al. (2001) evaluated the reproductive toxicity of DDT in adult male rats exposed to 50 and 100 mg/kg body weight (b.wt) day-1 for 10 successive days and concluded that DDT led to reduction of testicular weight and the number as well as the percentage of motile spermatozoa in the epididymis. Histological observations of the testicle revealed a marked loss of gametes in the lumen of seminiferous tubules. Hu and Wang (2008) showed the joint toxicity of phoxim (Pho) and fenvalerate (Fen) on the spermatogenesis of male rats. Phoxim and Fenvalerate jointly impaired spermatogenesis in a dose- and time- dependent manner. Their joint action exhibited a synergetic effect and increased toxicity.

4.1.3 Effects of organophosphate Insecticides on male reproductive system

Organophosphate pesticides (OPs) are phosphoric acid esters or thiophosphoric acid esters and are among the most widely used pesticides for insect control.
Dimethoate at 28 mg kg^{-1} day^{-1}, deltamethrin at 5 mg kg^{-1} day^{-1} and their mixture at 5 mg kg^{-1} day^{-1} were associated with a significantly decreased sperm count, motility and viability and significantly increased percent morphologically abnormal spermatozoa (Abdallah et al., 2010).
Subchronic exposure of male rat to dimethoate (2, 8 and 20 mg/kg for 90 days) induced a decrease in relative testis weights (Sayým, 2007). In light microscopic examinations, histopathological observation of treated rats revealed that dimethoate caused dose-related testicular damage characterized by moderate to severe seminiferous tubule degeneration as sloughing, atrophy, germ cell degeneration and by partial arrest of spermatogenesis. Farag (2007) demonstrated the adverse effects of dimethoate on the reproductive performance of male mice. The sperm viability, motility and density were reduced in dimethoate treated mice. Ngoula et al. (2011) also obtained similar results in male rats treated with Dimethoate. Testicular and epididymal sperm density were decreased in rats treated with malathion. Pre and post fertility test showed 80% negative results after treatment. Biochemical profile of the testis revealed a significant decline in the contents of sialic acid and glycogen. Whereas a significant increase in the protein content of testis and testicular cholesterol was observed. The activity of testicular enzyme acid phosphatase increased significantly, while decreased alkaline phosphatase activity was found (Choudhary et al., 2008).

Histopathological studies of the intoxicated rats (Treated with methomyl orally 17 mg/kg in saline daily for two months) revealed variable degrees of degenerative changes in the seminiferous tubules up to total cellular destruction (Mahgoub and EI-Medany, 2006).
A single injection of parathion (organophosphate agro pesticide) to immature male mice led to a decrease in testis weight and early damage of germ cells of the mice. The effect is reversible and recovers at longer intervals (Sobarzo and Bustos-Obregon, 2000). In adult Wistar rats orally treated with pirimiphos-methyl (41.67, 62.5 or 125 mg/kg) for 90 days, a decrease in relative testis and epidiydimis weights and intra-testicular cholesterol level was recorded. whereas a decrease in serum total protein, sperm density and motility, fertility and parturition indices and pups sex-ratio (M/F) was recorded in animals treated with 125 mg/Kg of pirimiphos methyl. Histological findings also indicated enlargement of interstitial space, inhibition of spermatogenesis, rarefaction of Leydig cells and oedema in testes of treated rats (Ngoula et al., 2007[b]).
A single injection of the organophosphorous agroinsecticide parathion (6.67 mg/kg bwt corresponding to 1/3 of LD_{50} dose) to immature male mice (upon the onset and installation of spermatogenesis in immature CF1 mice) led to a decrease of testis weight and an early damage of germ cells in treated mice. The effects are reversible and recover at long intervals (Sobarzo and Bustos-Obregón, 2000[b]).
Quinalphos a commonly used organophosphorus insecticide reduce prostatic acid phosphatase activity and fructose content of the accessory sex glands, and plasma levels of testosterone and FSH and LH (Rey et al., 1991) as well as relative weights of the testis and accessory sex organs. Dimethoate orally exposed to male rats increase relative weights of testis and prostate, sperm density and motility, serum and testis levels of protein and cholesterol, activity of prostatic acid phosphatase. Testicular and epididymal histology generally shown in the testis, spams of Sertoli cells destruction and disorganization of germinal epithelium and in the epididymis, the proliferation of epithelial cells. The lumen of seminiferous tubules and epididymis were generally poor in sperm (Ngoula et al., 2011).
Methyl parathion adversely affect male rat reproductive organs by inducing vacuolization of the epithelium of seminiferous tubules, nuclear pyknosis and brush border disruption in the ductus deferens with the presence of immature cells in the lumen. Also, the activity of acid phosphatase was reduced (Narayana et al., 2006). Methyl Parathion caused significant decrease in the weight of testis, epididymis, seminal vesicle and ventral prostate with marked pathomorphological changes. Also, marked reduction in epididymial and testicular sperm counts was observed in exposed male rats. Fertility test showed 80% negative fertility in treated animals. A significant reduction in the sialic acid contents of testis, epididymis, seminal vesicle, ventral prostate and testicular glycogen were noticed, while the protein and cholesterol content were raised significantly (Suresh at al.,2003).
Methyl Parathion orally administered to male rats at levels of 50, 150 and 250 mg/kg for 60 days reduced the weight of the testes, epididymis, seminal vesicle and ventral prostate. Testicular and epididymal sperm density were also decreased in the treated animals. Pre and post fertility test showed 80% negative results after treatment Choudhary et al. (2003).
Co- treatment of malathion-exposed rats with vitamins E and C had a protective effect on sperm counts and sperm motility. Degenerative changes in the seminiferous tubules were also observed in the rats which received malathion and supplemented with vitamins C and E, but milder histopathological changes were observed in the interstitial tissues (Uzun et al., 2009).

Body and testis weights decreased in methyl parathion (0.28 mg/kg b.wt per day for 7 weeks) treated rats. It was observed that, at the end of 4th and 7th weeks there was a statistically significant decrease in sperm counts and sperm motility, increase in abnormal sperm morphology Meltem et al. (2007). Joshi et al. (2007) investigated the effects of chlorpyrifos on testes. Chlorpyrifos methyl orally administered to male rats at the dose levels of 7.5, 12.5 and 17.5 mg/kg b. wt. /day for 30 days showed marked reduction in epididymal and testicular sperm counts in exposed males. Histopathological examination of testes showed mild to severe degenerative changes in seminiferous tubules at various dose levels. Fertility test showed 85% negative results.

4.1.4 Effects of pyrethroid Insecticides on male reproductive system

Pyrethrin is an extract from several types of chrysanthemum, and is one of the oldest insecticides used by humans. There are six esters and acids associated with this botanical insecticide. Pyrethrin is applied at low doses and is considered to be nonpersistent. Mammalian toxicity of pyrethrins is quite low, apparently due to its rapid breakdown by liver microsomal enzymes and esterases. Exposure to the higher concentration of cypermethrin disturbed the reproductive behaviour of the parr. They displayed fewer courting events, spent less time near the nesting females and had lower volumes of strippable milt. They also had significantly lower amounts of 11-ketotestosterone (11-KT) in the blood plasma. Further, in control fish, higher plasma levels of 17,20-P were observed in parr interacting with a female compared to those with no female contacts (Jaensson et al., 2007).

Yao & Wang (2008) observed a new type of pesticides and because of their high performance and low toxicity, pyrethroid insecticides are widely used in place of organochlorine insecticides both in agriculture and in the home. Recent researches indicates that pyrethroid insecticides can reduce sperm count and motility, cause deformity of the sperm head, increase the count of abnormal sperm, damage sperm DNA and induce its aneuploidy rate, as well as affect sex hormone levels and produce reproductive toxicity. Meeker et al. (2008) reported reduced semen quality and increased sperm DNA damage in relation to urinary metabolites of pyrethroid insecticides.

4.2 Effects of insecticides on female reproductive system

Insecticides that target the female reproductive system can cause a wide variety of adverse effects. Changes in sexual behavior, onset of puberty, cyclicity, fertility, gestation time, pregnancy outcome, and lactation as well as premature menopause are among the potential manifestations of female reproductive toxicity: all can disrupt a female reproduction.

4.2.1 Effects of organochlorine insecticides on female reproductive system

Residue levels of chlorinated insecticides continue to be found in the environment and, although the concentrations are low approaching the limit of delectability, there stil play a big role. Organochlorine compounds are known to interrupt the estrus cycle in rats (Martinez and Swartz, 1991; Uphouse et al, 1984; Swartz and Mall, 1989).

Chronic treatment of young female rats with 5, 10, 20, and 40 mg/kg lindane delayed vaginal opening and disrupted ovarian cyclicity up to approximately 110 days of age. Thereafter, regular ovarian cycles were present in the majority of females (Ralph et al., 1989). In addition, exposure of mink to Lindane from conception resulted in a decrease in

reproductive efficiency when they were subsequently mated, leading to a 60% reduction in the number of kits born (Beard and Rawlings, 1998). Acephate treatment was associated with a decreased number of implantations and live fetuses, and an increased number of early resorptions at 28 mg/kg/day (Farag et al., 2000).

Study on infertile German women found association between endometriosis and elevated levels of chlorinated hydrocarbon pesticides (Gerhard et al., 1999). However, no association was found in wives of pesticide applicators in Minnesota, or with levels of chlorinated hydrocarbon pesticides in infertile women in Canada (Lebel et al., 1998).

A study done in Florida at a time of heavy application of DDT in agricultural showed that 14 ppb concentration level was found in black babies and 6 ppb in whites. However, other studies have found low levels of DDE and hexachlorocyclohexane in California women in their second trimester of pregnancy (Moses, 1995). The presence of pesticides in cord blood is evidence of transplacental passage. Most tests of maternal/fetal pairs are for persistent pesticides in the DDT family. The highest reported level of DDE was found in Mexican babies born in 1997 (4700 ppb), and DDT levels were also higher (880 ppb). The highest level of hexachlorobenzene (HCB) was reported in 1985 from Tunisia (37 ppb) while the lowest levels were found in babies in Nicaragua (6.39 ppb), Spanish babies born between 1997 to 1999 (1.1 ppb) and German babies born in 1994 (0.5 ppb). Higher levels of DDT and lindane were found in stillbirt babies in India but the levels were not significant from full term births babies (Sancewicz-Pach et al., 1997).

Chlordecone (0.015, 0.03, 0.06 and 0.125 mg in 0.05 ml sesame oil) intra pertitonally injected 10 times during 12 days to one-day-old female mice may produce distinct morphological alterations in the epithelium lining both the vagina and uterus. The changes in the neonate mouse reproductive tract appeared dose related in that increased doses of administered chlordecone accelerated development of the vaginal epithelium leading to keratinization while cellular hypertrophy, hyperplasia, and glandular formation were observed in the uterus. These changes appeared identical to the developmental changes induced by the estradiol (Eroschenko and Moussa, 1979).

Treatment of immature rats with chlordane, dieldrin, heptachlor, lindane, p,p'-DDT, p,p'DDE, or toxaphene for 7 days stimulates the metabolism of estrone by liver microsomal enzymes and inhibited the increase in uterine wet weight caused by estrone (Welch et al., 1971). Another study in German found no significant differences in the levels α-β-γ isomers of hexachlorcyclohexane, heptachlor, dieldrin, and total DDT in the subcutaneous fat of children who died of Sudden Infant Death Syndrome (SIDS) compared to children who died of known causes (Kleemann et al. 1991).

The pesticide heptachlor may cause disrupted and prolonged estrus cycles (Oduma et al., 1995). Treatment with DDT and chlordecone resulted in persistent estrus in rats. Lindane induced marked disturbances in the estrus cycle, prolonging the proestrus phase considerably and thereby delaying ovulation (Chadwick et al., 1988; Pages et al., 2002, Lahiri et al., 1985).

4.2.2 Effects of organophosphate insecticides on female reproductive system

No reports on organophosphate pesticides in cord blood were found. Studies in California and Florida found decreased cholinesterase activity, a biomarker of organophosphate exposure. Since these pesticides are not persistent, the findings reflect recent exposure. Monocrotophos, an organophosphate insecticide, administered to female rats provoked

embryonic resorptions. Fertility and parturition indices were reduced in dose dependent fashion. However, gestation index was not affected. Viability and lactation indices were highly reduced in rats of high dose group. Birth weight and crown-rump length of pups in high dose group were significantly less, with no effect on average litter size (Adilaxmamma et al., 1994).

Methyl Parathion (MP) (oral gavage for five days a week for four weeks at a daily dose) led to delections in microvilli and Marked loss in kinocillia of surface epithelium of fallopian Tube (Mehmet et al., 2007). In addition, the number of estrus cycles and the duration of each phase of the estrus cycle were significantly affected after treatment of rats with methyl parathion (Asmathbanu and Kaliwal, 1997; Dhondup and Kaliwal, 1997). The pesticides dimethoate, malathion, and sumithion gave similar results (Kumar and Uppal, 1986; Gouda and Sastry, 1979)

4.2.3 Effects of pyrethroid insecticides on female reproductive system

Permethrin exposure has caused embryo loss in pregnant rabbits (US EPA, 1997) and in pregnant rats (Spencer and Berhane, 1982). Moreover, in pregnant rabbits, feeding of cyfluthrin causes both miscarriages and resorption of fetuses. In a three-generation study of rats, feeding of cyfluthrin caused pups to have "decreased viability" and decreased weight (US EPS, 1988). Cyfluthrin may also have more subtle effects on the ability of humans and other animals to reproduce. The researchers advise protection from any form of contact or ingestion of the pyrethroids in order to prevent any undesirable effects on the human reproductive syste (Eil et al., 1990).

In sexually mature female rats orally intubated with the organophosphorus insecticide, Pestban at a daily dosage of 7.45 or 3.72 mg/kg bwt. respectively for 14 days during premating, mating and throughout the whole length of gestation and lactation periods showed reduced fertility with increasing the dose. In addition, the number of implantation sites and viable fetuses were reduced in pregnant females. However, the number of resorptions, dead fetuses, and pre-and postimplantation losses were increased. The behavioral responses as well as fetal survival and viability indices were altered during the lactation period (Morgan, 2008).

4.2.4 Effects of other insecticides or mixture of insecticides on female reproductive system

A single dose of Frontline approximately doubled the time between periods of estrus in female rats (Ohi et al., 2004). Higher doses of Frontline also reduced the number of female rats who were able to become pregnant following mating (Ohi et al., 2004). Offspring of rats fed fipronil during pregnancy were smaller than offspring of unexposed rats. In addition, male offspring from exposed mothers took longer than offspring of unexposed mothers to mature sexually. These effects occurred at all but the lowest dose level tested. In another study, fipronil reduced litter size, fertility, and the survival of offspring. These effects occurred at the highest dose level tested (U.S. EPA, 1998[1]). Offspring of rats exposed to fipronil had smaller brains than the offspring of unexposed rats. In addition, the fipronil exposure caused a variety of behavioral changes. All of these effects occurred at the highest dose level tested (U.S. EPA, 1997 and 1998).

Carbofuran affected the estrus cycle by showing a decrease in the number of estrus cycles and the duration of each phase, which may be due to a direct effect on the ovary or on the

hypothalamus-pituitary-ovarian axis causing hormonal imbalance (Baligar and Kaliwal, 2002).
Another pesticide found in cord blood is the widely used insect repellent deet. In a study in Thailand deet was found in 8% of babies whose mothers used the repellent in the second and third trimester of pregnancy (Moses, 1995).

4.3 Effects of Insecticides on endocrine system

The fate and detoxification of organochemicals have not been well defined, but these agents can disrupt the hypothalamic-pituitary-tesicular axis affecting the endocrine and reproductive functions. Since environmental exposure is due to a mixture of various endocrine disruptors, the effect of their combined toxicity becomes more important.
Development of a fetus into a phenotypic male depends, first, on testis formation and second, on hormone production by the fetal testis. Disorders of testicular hormone production or action can lead in severe cases to phenotypic abnormalities or can predispose towards impaired reproductive health. Though it is concluded that no direct evidence links human exposure to environmental chemicals and male reproductive disorders that stem from disturbed testis development, this is based mainly on lack of information (Sharpe, 2001).

4.3.1 Effects of organochlorine insecticides on endocrine system

Organochlorine insecticide Lindane lower serum testosterone levels in animals, block steroid hormone biosynthesis in leydig cells by reducing StAR protein expression (Walsh et al., 2000; Walsh et Stocco, 1998). DDT, a commonly used pesticide, and its metabolites (o,p-DDT, and p,p'DDE) have estrogenic effects in males by blocking the androgen receptor (Whorton et al., 1977; Mattison, 1983; Kelce et al., 1995). DDT inhibited the cAMP response to follicle-stimulating hormone (FSH), the major endocrine control of Sertoli cell development, and to a β2-agonist, isoproterenol. DDT exposure decreased the level of FSH binding sites. The DDT inhibitory effect on the FSH response was also observed in Ser W3 cells, a Sertoli cell-derived immortalized cell line (Bernard et al., 2007). DDE, a metabolite of DDT, has anti-androgenic action and can also jeopardize estrogen metabolism in its synthesis or breakdown and physiological elimination (Toppari, 1996; Kelce, 1995). DDE may also inhibit the expression of transferin (Tf) and upmodulate expression of ABP in cultured rat Sertoli cells (XianZhi et al., 2006).
Young females rats exposed to Lindane (20 and 40 mg/kg) presented smaller pituitary and uterine weights, lower serum and pituitary luteinizing hormone (LH) and prolactin, serum estrogen concentrations and higher pituitary follicle stimulating hormone (FSH) concentrations. Thus, indane may effectively block the response of estrogen-dependent tissues to this ovarian steroid hormone and that this apparent antiestrogenic effect of lindane is responsible for the disturbances observed in the neuroendocrine control of ovarian function in the rat (Cooper et al., 1989). It is shown that chronic administration of lindane results in endocrine disruption in birds as well as in mammals (Saradha et al. 2009). Treatment with 1-40 mg of lindane/kg body weight, inhibited testicular steroidogenesis, reduced plasma androgen concentrations and impaired reproductive performances in males (Page et al., 2002).
Toxic manifestations of dermally applied hexachlorocyclohexane (50 mg or 100 mg kg-1 body weight day-1, 5 days in a week for 120 days) on testes led to a decrease in serum testosterone levels Prasad et al. (1995).

2,3,7,8-Tetrachlorodibenzo-p-Dioxin (TCDD) can have an anti-androgenic and anti-estrogenic effect (Eskenazi and Kimmel, 1995), inducing a decrease in the testicular response to LH (Eskenazi and Kimmel, 1995; Bush et al., 1996).

4.3.2 Effects of organophosphate insecticides on endocrine system

Quinalphos a commonly used organophosphorus insecticide reduce prostatic acid phosphatase activity and fructose content of the accessory sex glands, and plasma levels of testosterone and FSH and LH (Rey et al., 1991) as well as relative weights of the testis and accessory sex organs. Thus, Quinalphos exert suppressive effects on the functional activity of accessory sex glands by decreasing testicular testosterone production following inhibition of pituitary gonadotrophins release (Rey et al., 1991). Quinalphos also reduce prostatic acid phosphatase activity and fructose content of the accessory sex glands, and plasma levels of testosterone and FSH and LH.

Chlorpyrifos orally administered to male rats at the dose levels of 7.5, 12.5 and 17.5 mg/kg b. wt. /day for 30 days showed a decrease in serum testosterone concentration (Joshi et al., 2007). Treatment of rats with the insecticide heptachlor suppressed estradiol concentrations in blood and reduced the production of estradiol by ovarian cells of treated rats (Oduma et al., 1995; Rami et al., 1995). Lindane, also cause a decrease in circulating estradiol levels in rats (Eldridge et al., 1994; Gojmerac et al., 1996). In monkeys, ovulatory levels of estradiol were reduced after high doses of hexachlorobenzene (Foster et al., 1995), which also induced anovulatory cycles and suppression of circulating levels of estradiol (Muller et al., 1978), and a dose dependent suppression of serum progesterone concentrations during the luteal phase (Foster et al., 1992). Progesterone levels may be decreased by exposure to methoxychlor as well, especially during the estrus phase of the estrus cycle in rats (Chapin et al., 1997; Cumming and Lasley, 1993). During early pregnancy, progesterone concentrations decreased after treatment with DDT in rabbits (Lindeneau et al., 1994).

4.3.3 Effects of carbamate insecticides on endocrine system

Methomyl, a Carbamate insecticide administered to male rat daily for 65 successive days at two doses (0.5 and 1 mg/kg body weight) significantly decreased serum testosterone level (Shalaby et al., 2010).

Subchronic exposure to *methomyl (Carbamate)* induce a significant decrease in the level of *testosterone* in the intoxicated rats, while the levels of FSH, LH and *prolactin* significantly increased (Mahgoub and EI-Medany, 2006). The hormonal changes and testicular damage continued for 30 days after withdrawal of the insecticide indicating a persistent effect (Mahgoub & EI-Medany, 2006). Subchronic exposure of male rats to methomyl also provoked a decrease in the level of testosterone (Choudhary et al., 2008; Afaf et al., 2000), while the level of FSH, LH and prolactin increase (Afaf et al., 2000). The rats given malathion alone or in combination with vitamins also had lower plasma FSH, LH and testosterone levels than the control rats (Uzun et al., 2009).

4.3.4 Effects of pyrethroid insecticides on endocrine system

Experimental studies have reported that pyrethroid insecticides affect male endocrine and reproductive function, but human data are limited. Serum reproductive hormones levels of 161 men recruited from an infertility clinic as well as the pyrethroid metabolites 3-phenoxybenzoic acid (3PBA) and cis- and trans-3-(2,2-dichlorovinyl)-2,2-

dimethylcyclopropane carboxylic acid (cis-DCCA and trans-DCCA) in spot urine samples were determined. When adjusting for potential confounders, categories for all three metabolites, as well as their summed values, were positively associated with FSH. Suggestive positive relationships with LH were also found. In addition, cis-DCCA and trans-DCCA were inversely associated with inhibin B (p for trend=0.03 and 0.02, respectively). Finally, there was evidence that trans-DCCA was inversely associated with testosterone and free androgen index (Meeker et al., 2009).
Wistar rats received daily (po), from day 6 of pregnancy to day 21 of lactation, deltamethrin (D) and endosulfan (E) concomitantly: D: 2.0 mg/kg + E: 1.5 mg/kg, or D: 3.0 mg/kg + E: 2.0 mg/kg, or D: 4.0 mg/kg + E: 3.0 mg/kg. Results from the uterotrophic assay indicate absence of in vivo estrogenic activity of D + E. No significant variations in reproductive endpoints of females were observed (Kenia et al., 2005).
Permethrin affects both male and female reproductive systems. It binds to receptors for androgen, a male sex hormone, in skin cells from human males (Eil and Nisula, 1990). Permethrin also binds to a different receptor, called the peripheral benzodiazepine receptor, which stimulates production of the male sex hormone testosterone (Ramadan et al., 1988). Cyfluthrin also binds with peripheral benzodiazepine (PBZ) receptors. PBZ receptors are found in high concentration in the testes and appear important in "hormonal responsiveness (Ramadan et al., 1988).
A research documented the ability of six synthetic pyrethroids, as well as the naturally occurring pyrethrins, to bind with androgen (a male sex hormone) receptors, and disrupt normal androgen function (Eil et al., 1990).

5. Developmental toxicity of insecticides

Developmental toxicity conferred to any structural or functional alteration or perturbation, caused by environmental insult, reversible or irreversible, which interferes with homeostasis, normal growth, differentiation, development and/or behaviour (Rochelle, 1988). Developmental abnormalities constitute a significant medical problem and greatly contribute to animal and human suffering. Protective barrier of placenta is not always enough to shield the developing embryo or foetus from chemical exposure via the mother. This chemical can be toxic, lethal or cause birth defects in the developing embryo or foetus. The developmental process is particularly vulnerable to adverse environmental conditions including chemical pollutants such as insecticides which have been ubiquitous because of its widespread manufacture, and disposal all over the world. They have direct effects resulting in impaired fertility, high rates of abortions, and abnormal pregnancies. In fact, every developmental stage is vulnerable to any environmental insult which encompasses a spectrum of possible effects which includes malformation of fertilized egg or zygote, of the embryo during organogenesis, the foetus in the post embryonic period of gestation and the postnatal until sexual maturity of offspring.

5.1 Effects of insecticides on fertilization

Incubation of sea urchin *Paracentrotus lividus, eggs* for 1 h in the presence of increasing concentrations of lindane, methoxychlor, or dieldrin up to 100μM, rinsed in filtered sea water and then fertilized with a final 10^4 -fold sperm dilution led to the decrease of fertilization rate. Treatment of eggs with each pesticide did not prevent fertilization, but increased the rate in polyspermy, delayed or blocked the first mitotic divisions, and altered

early embryonic development. Moreover, all pesticides could alter several intracellular biochemical pathways that control first mitotic divisions and early development, including intracellular calcium homeostasis, MPF (mitosis promoting factor) activity and formation of the bipolar mitotic spindle. Lindane was the most potent of the three pesticides (Pesando et al., 2003). Dieldrin, methoxychlor, and lindane, can alter oocyte maturation in mammals and in marine invertebrates. The effects were observed at relatively high doses of pesticides (Picard et al., 2003).

The reproductive process ranges from the development and maturation of both female and male reproductive systems, to successful mating and a resulting healthy, normal viable offspring. Exposure of an organism to these chemicals can cause damage to the spermatogonial cells which represents male genome or cause damage to spermatozoid undergoing maturation (Oakes et al., 2002). These damages to spermatogenesis would lead to increased adverse fertilization effects when the males mated with females. The rapidly dividing spermatogonia are susceptible to toxicity induced by insecticides which affect cells division.

Some insecticides have been reported to have weak steroid activities at the level of the ovary, attenuating its sensitivity to gonadotropins and altered sperm motility in the oviduct (Khan-Dawood and Satyaswaroop, 1995). According to Blomqvist et al. (2005), roosters exposed to the chlorinated insecticide DDT and its persistent metabolites during their embryonic development resulted in persistent effects on epididymal-testicular structure and function had a significantly reduced semen production in adult stage.

5.2 Effects of insecticides on prenatal development

Many organotin compounds which are widely used in agriculture and industry have biocidal properties and are used in agriculture as insecticides. Their widespread use has caused increasing amounts to be released into environment. Exposure of an organism to insecticides at any time during foetal life can produce structural defects in developing organ systems such as the kidneys, the nervous system, and the skeleton. At this time, exposure to insecticides may also cause cancer in the prenatal or postnatal periods, altered growth, functional deficits, and prenatal death or premature senescence (Michal et al., 1993). These phenomena may result from interference with the endocrine system (Ema and Harazono, 2000) which disturb hormonal regulation during pre and postnatal development.

During embryonic stage of development, the conceptus is very vulnerable to environmental insult including insecticides because of differences from adult include the following:

- The organismic plasticity of small cell numbers found in the pre-embryonic stage of development is lost with the transition from presumption to determined cell status.
- Interference of rapid rates of cell proliferation during embryonic stage with the process of rapid synthesis of energy sources such ATP and GTP may preclude normal differentiation and growth, and may translate into growth retardation or malformation.
- The limited metabolic capability to produced enzymes responsible detoxifying xenobiotics.
- Lack of recognition capability of immunosurveillance systems due to the immaturity of embryonic cells as compared to postnatal cells with altered surface markers which can easily distinguish “self” and “non self”.

The finding of Blomqvist *et al*. (2005) showed that embryonic exposure to DDT or EE_2 (17alpha-ethynyl estradiol) induced a persistent effect on testicular function, and impaired fertility because of the reduced output of spermatozoa. Insecticides developmental delays in prenatal development is detected by reduced foetal or neonatal body weights in mammals, reduced absolute and relative organ weights, and reduced ossification of skeletal elements in foetus which it is conventionally evaluated during gestational day 20 or 21 in rats, day 17 or 18 in mice, and day 29 or 30 in rabbits (Rochelle, 1988). Due to totipotent cells which comprise the conceptus during pre-embryonic stage of development, which begins with fertilization and ends at implantation, and lasts approximately 5 to 8 days in most mammals, the conceptus is considered relatively refractory to the chemical compounds used in agriculture.

Chlorpyrifos-methyl (CPM) exhibit weak reproductive toxicity in F0 rats exposed at adulthood and negligible effects in F1 offspring exposed in utero and via lactation at weanling, but induce anti-androgenic effect and hypothyroidism after long term exposure from in utero through sexual maturation of F1 rats (Sang-Hee et al., 2006).

A study conducted by Rope et al. among male workers who were exposed to various mixtures of pesticides such as DDT, BHC, endosulfan; and organophosphorus pesticides i.e. malathion, methyl- parathion, dimethote, monocrotophos, phosphamidon and quinalphos; synthetic pyrethroids such as fenvelrate and cypermethrin during mixing and spraying showed male mediated adverse reproductive outcome such as abortion, stillbirths, neonatal deaths, congenital defects, etc. (Rupa et al., 1991).

5.3 Effects of insecticides on postnatal development

In some developing countries, women from the majority of the agricultural work force bringing them into contact with uncontrolled use of pesticides and other chemicals used in agriculture which may adversely affect reproduction and increase contamination of breast milk (Michal et al., 1993). There are many pathways for exposure: in drinking water from contaminated wells, in food, from household insecticide use, from residues on plants as they are picked or on machinery as it is being handled or repaired, from insecticide drift as it is being sprayed, from spills during transport and from dermal exposure during mixing or application. The paper by Nurminen et al. (1995) in the issue of epidemiology of birth defect from women exposure to insecticides revealed 95% of chance for the risk of having a child or stillborn infant with a structural birth defect among women employed in agricultural work.

Embryonic and postnatal exposure to high doses of insecticides like DDT and its derivatives induced a significant reduction in the average area of the seminiferous tubules of the male testis, indicating that an increased amount of interstitial tissue in the testis accompanies the decrease in tubular area (Blomqvist et al., 2005). A positive correlation between the diameter of the seminiferous tubules and sertoli cell size has been observed in Syrian hamster (Hikim et al., 1989), and experiments in rats have shown that sertoli cell development is modulated by estrogen (Sharpe et al., 1998). This report is supported by the finding that rats neonatally exposed to estrogens had fewer sertoli cells and a decreased diameter of the seminiferous tubules (Aceitero et al., 1998; Atanassova et al., 1999). Testicular deformations such as abnormal shape and blisters and a stunted epididymis were also reported by the finding of Blomqvist et al. (2005) on domestic rooster embryonically exposed to DDT. These effects were mainly seen in the left testis which has an ambisexual potential and is more sensitive to estrogen. As shown in gulls,

quail, and chicken (Fry and Toone, 1981; Berg et al., 1998), exposure to insecticides causes feminization of the left testis in bird embryo.
In contrast to testis, ovotestis formation appears to be a transient effect of embryo exposure to estrogenic substances. Several earlier studies on quail, and rooster embryonically exposed to insecticides are in agreement that ovotestis did not persist until adulthood (Scheib, 1983; Halldin et al., 1999). Likewise, quail exposed embryonically to 150 µg DDT/g egg did not show ovotestis as adults (Halldin et al., 2003).
Insecticides developmental delays in postnatal offspring may be indicated by reduced body weight or weight gain, considered a sensitive and consistent measure of developmental effect (Rochelle, 1988).

6. Perspectives of research in developmental and reproductive toxicity of insecticides

Expensive studies that are able to incorporate direct exposure assessments on large populations are needed. Before this can be justified as other studies have suggested (Rowland, 1995), the basic question which needs further attention is to know if "people who perform agriculture work at increased risk"? There are substantial differences in the ways insecticides are used within different types of farming that may not have been adequately appreciated in the past. Collaboration between registries and pooling of cases when case-control studies are planned is worth considering. Since adverse reproductive outcome may be an indirect measure of the exposure effect, it is urgent to rethink what has long been standard approach to modelling toxicity of environmental insults and develop alternative methods for doing more quantitative-based insecticides exposure assessments. To incorporate these methods into more accurate exposure assessments in case-control studies of reproduction defects and insecticides, epidemiologists and industrial hygienists will need to work together to characterize determinants of insecticide exposure that are region and crop specific.

7. References

Abdallah, F. B., Slima, A. B., Dammak, I., Keskes-Ammar, L. and Mallek, Z. Comparative effects of dimethoate and deltamethrin on reproductive system in male mice. *Andrologia* 2010; 42: 182–186.

Aceitero, J., Llanero M., Parrado, R., Pena, E., Lopez-Beltran, A. Neonatal exposure of male rats to estradiol benzoate causes rete testis dilation and backflow impairment of spermatogenesis. *Anatomy Research*, 1998; 252(1): 17-23.

Adilaxmamma, K., Janardhan, A. and Keddy, K.S. Monocrotophos: Reproductive toxicity in rats. *Indian Journal of Pharmacology* 1994; 26: 126-129.

Alegakis, A. K., Koumantakis, E., and Troulakis, G. Experiences with acute organophosphate poisonings in Crete. *Vet. Hum. Toxicol.* 1996; 38(2): 101-107.

Amer, S.M. and Aly, F.A. Genotoxic effect of 2, 4-dichlorophenoxy acetic acid and its metabolite 2,4- dichlorophenol in mouse. *Mutat. Res.* 2001; 494: 1-12.

Andersen, A.G., Jensen, T.K., Carlsen, E., Jorgensen, N., Andersson, A.M., Krarup, T., Keiding N, et Skkaek, N.E. Fréquence élevée de qualité de sperme inférieure à la moyenne chez une population non sélectionnée d'hommes jeunes: *Reproduction Humaine* 2000; 15 (2): 366-372.

Asmathbanu, I, Kaliwal, B.B. Temporal effect of methyl parathion on ovarian compensatory hypertrophy, follicular dynamics and estrous cycle in hemicastrated albino rats. *J Basic Clin Physiol Pharmacol* 1997; 8:237-254.

Assunção, J.V., Pesquero, C.R. Dioxinas e furanos: origens e riscos. *Rev Saúde Pública* 1999; 33:523-30.

Atanassova, N., McKinnell, C., Walker, M., Turner, K.J., Fisher, J.S., Morley, M., Millar, M.R., Groome, N.P., and Sharpe, R.M,. Permanent effects of neonatal estrogen exposure in rats on reproductive hormone levels, Sertoli cell number and the efficiency of spermatogenesis in adulthood. *Endocrinology* 1999; 140: 5364-5373.

Aziz, N, Walliullah, S.S. and Aziz, R.N. Histological Changes in Male Rat Reproductive Organs Post-Treated With Insecticide Carbofuran (Furadan). *Annals of Microscopy* 2008 ; 8 : 83-89.

Baligar, P.N., Kaliwal, B.B. Reproductive toxicity of carbofuran tothe female mice: effects on estrous cycle and follicles. *Ind Health* 2002; 40:345-352.

Barrett, K.E., Barman, S.M., Boitano, S. and Brooks, H.L. Ganong's Review of medical physiology 23rd Edition McGraw Hill 2010.

Beard, A. P. and Rawlings, N. C. Reproductive effects in mink (Mustela vison) exposed to the pesticides Lindane, Carbofuran and Pentachlorophenol in a multigeneration study. *Journal of Reproduction and Fertility* 1998; 113, 95-104.

Bell, R., Sally, C., Ming, Q., Alwyn, F., Paul, M. F., Tao, J., George, L., Alan, M.N., Brian, G., Rose, L., Tran, k. and Shaun, W.: Toxicity of 2,3,7,8- tetrachlorodibenzo-p-dioxin in the developing male Wistar (han) rat. I: no decrease in epididymal sperm count after a single acute dose. *Toxicological Sciences* 2007; 99: 214–223.

Ben Rhouma, K., Tebourbi, O., Krichah, R.N. & Sakly, M. Reproductive toxicity of DDT in adult male rats. *Human Experimental Toxicology* 2001; 20 (8): 393-397.

Blomqvist, A., Berg, C., Holm, L., Brandt, I., Ridderstrale, Y., & Bruneträm, B. Defective reproductive organ morphology and function in domestic Rooster embryonically exposed to O,P'-DDT or ethynylestradiol. *Biology of Reproduction* 2006; 74(3): 481-486.

Bush, B., Lambert, G., Tarbell, A. Polychlorinated biphenyl (PCB) and dichlorodiphenyl dichloroethylene (DDE) exposure among Native American men from contaminated Great Lakes fish and wildlife. *Toxicol Ind Health* 1996; 12:361-8.

Cabello, G., Valenzuela, M. & Vilava, A. A rat mammary Tunor Model Induced by the Organophosphorous pesticides Parathion and Malathion, possibly through Acetylcholinesterase inhibition. *Environmental Health perspectives* 2001; 109 (5): 471-479.

Campbell, N.A. & Reece J.B. Biology 7th Edition, Binjamin Cummings, San Francisco, Boston, New York 2005; 1231p.

Chadwick, R.W., Cooper, R.L., Chang, J., Rehnberg, G.L., McElroy, W.K. Possible antiestrogenic activity of lindane in female rats. *J Biochem Toxicol* 1988; 3:147-158.

Chapin, R.E., Harris, M.W., Davis, B.J., Ward, S.M., Wilson, R.E., Mauney, M.A., Lockhart, A.C., Smialowicz, R.J., Moser, V.C., Burka, L,T., Collins, B.J. Theeffects of perinatal/juvenile methoxychlor exposure on adult rat nervous, immune, and reproductive system function. *Fundam Appl Toxicol* 1997; 40:138-157.

Choudhary, N., Goyal, R. & Joshi, S.C.. Effect of malathion on reproductive system of male rats. *Journal of Environmental Biology* 2008; 29(2): 259-262.

Choudhary, N., Goyal, R. and Joshi, S.C. Reproductive toxicity of endosulfan in male albino rats. *Environ. Contam. Toxicol* 2003; 70:285–289.

Chung, M.K., Kim, J-C., Han, S.S. Developmental toxicity of flupyrazofos, a new organophosphorus insecticide, in rats. *Food and Chemical Toxicology* 2002; 40: 723–729.

Clark, G., Garry, V., Burroughs, B., Holland, S., Chu, M. & Brown, D. Endocrine disruptors. Relationships between exposure to dioxin-like chemicals, testosterone levels, and sex of the children of pesticide applicators. *Organohalogen Compounds* 2002; 56: 73-76.

Colborn, T., Myers, P.J. & Dumanoski, D. Our Stolen Future. Penguin Boocks, USA Inc., New York. Consensus d'actualisation SFAR - Médecine d'Urgence 1996; 18 p.

Colborn, T., Vom Saal, F.S. & Soto, A.M. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environmental Health Perspectives* 1993; 101: 378-384.

Cooper, R.L., Chadwick, R.W., Rehnberg, G. L., Goldman, J.M., Booth, K.C., Hein J.F. & W. Keith McElroy. Effect of lindane on hormonal control of reproductive function in the female rat. *Toxicology and Applied Pharmacology* 1989; 99(3) : 384-394.

Corwin, E. J. Handbook of Pathophysiology, 3rd Edition, Lippincott Williams & Wilkins 2008.

Cummings, A.M., Laskey, J. Effect of methoxychlor on ovarian steroidogenesis: role in early pregnancy loss. *Reprod Toxicol* 1993; 7:17-23.

Darren, M.R., Ayanthi, K., Nick, A.B., Gamini, M,H., Sheriff, R. & Eddleston, M. Influence of pesticide regulation on acute poisoning deaths in Sri-Lanka. *Bulletin of World Health Organization* 2003; 81: 112-120.

Delemarre-van de Waal, H.A. Environmental Factors Influencing Groth and Pubertal Development. *Environmental Health Perspectives* 1993; Supplément 101. 39-44.

Dhondup, P., Kaliwal, B.B. Inhibition of ovarian compensatory hypertrophy by the administration of methyl parathion in hemicastrated albino rats. *Reprod Toxicol* 1997, 11:77-84.

Eil, C. and Nisula B.C. The binding properties of pyrethroids to human skin fibroblast androgen receptors and to sex hormone binding globulin. *J. Steroid Biochem*. 1990; 35(3/4):409-414.

Eldridge, J.C., Fleenor-Heyser, D.G., Extrom PC, Wetzel, L.T., Breckenridge, C.B., Gillis, J.H., Luempert, L.G. III, Stevens, J.T. Short-term effects of chlorotriazines on estrus in female Sprague-Dawley and Fischer 344 rats. *J Toxicol Environ Health* 1994; 43:155-167.

Eroschenko, V.P. & Mousa, M. A. Neonatal administration of insecticide chlordecone and its effects on the development of the reproductive tract in the female mouse. *Toxicology and Applied Pharmacology* 1979 ; 49 (1) : 151-159.

Eskenazi, B., Kimmel, G. Workshop on perinatal exposure to dioxin-like compounds. II. Reproductive effects. *Environ Health Perspect* 1995; 103:143-5.

Faqi, A.S., Dalsenter, P.R., Ligensa, A., Merker, H.J. and Chahoud, I.: Effect on male fertility and testis concentrations of low dose of 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD) in offspring of rats exposed during pregnancy and lactation. *Teratology* 1997; 56: 403.

Farag, A. T., Ahmed, F., Aswad, E. and. Shaaban, N. A.: Assessment of reproductive toxicity of orally administered technical dimethoate in male mice. *Reprod. Toxicol* 2007; 23: 232-238.

Farag, A.T., Eweidah, M.H., El-Okazy, A.M. Reproductive toxicology of acephate in male mice. *Reprod Toxicol* 2000; 14(5):457-462.

Farag, AT, Eweidah, MH, Tayel, SM, El-Sebae, AH. Developmental toxicity of acephate by gavage in mice. *Reproductive Toxicology* 2000; 14: 241–245.

Foster, W.G., McMahon, A., Villeneuve, D.C., Jarrell, J.F. Hexachlorobenzene (HCB) suppresses circulating progesterone concentrations during the luteal phase in the cynomolgus monkey. *J Appl Toxicol* 1992; 12:13-17.

Foster, W.G., McMahon, A., Younglai, E.V., Jarrell, J.F., Lecavalier, P. Alterations in circulating ovarian steroids in hexachlorobenzeneexposed monkeys. *Reprod Toxicol* 1995; 9:541-548.

Gerhard, L., Monga, B., Krahe, J., Runnebaum, B. Chlorinated hydrocarbons in infertile women. *Environ Res* 1999; (80-4) : 299-310.

Giwercman, A., Carlsen, E., Keiding, N., Skakkebaek, N.E. Evidence for increasing incidence of abnormalities of the human testis: a review. *Environ Health Perspect* 1993; 101:65-71.

Gojmerac, T., Kartal, B., Curic, S, Zuric, M., Kusevic, S., Cvetnic, Z. Serum biochemical changes associated with cystic ovarian degeneration in pigs after atrazine treatment. *Toxicol Lett* 1996; 85:9-15.

Gouda, H., Sastry, M.S. Effect of 0,0-Dimethyl-0 phosphorothicate (sumithion) on reproductive performance in rats and oestrogenic activity in mice. *Indian J Pharmacol* 1979; 11:287-292.

Hakin, L.S., Oates, R.D. Non-surgical treatment of male infertility: specific therapy. In: Lipshultz LI, Howards SS, editors. Infertility in the male. St. Louis: Mosby Year Book; 1997. p. 395-402.

Halldin, K., Berg, C., Brandt, I.& Brunström, B. Sexual behaviour in Japanese quail as a test end point for endocrine disruption: effects of in ovo exposure to exposure to ethinylestradiol and diethylstilbestrol. *Environ Health Perspect* 1999; 107: 861-866.

Halldin, K., Holm, L., Ridderstrale, Y., & Brunström B. Reproductive impairment in Japanese quail (Corturnix japonica) after in ovo exposure to O'P'-DDT. *Archive of Toxicology* 2003; 77: 116-122.

Hikim, A.P., Amador, A.G., Klemcke, H.G., Bartke, A.& Russell, L.D. Correlative morphology and endocrinology of Sertoli cells in hamster testes in active and inactive states of spermatogenesis. *Endocrinology* 1989; 125: 1829-1843.

Hodgson, E. A textbook of Modern Toxicology 3rd Edition A John Wiley&Sons Publication 2004.

Hwa–Young, S., Yong–Bum, K., Boo–Hyon, K., Sung–Whan, C. Chang–Su, H., & Jung–Koo R.. Effects of 2-bromopropane on spermatogenesis in the sprague–dawley rat. *Reproductive Toxicology* 1999; 13 (3): 179–187.

Jaensson, A., Scott, A.P., Moore, A., Kylin, H. & Olsen, K.H. Effects of a pyrethroid pesticide on endocrine responses to female odours and reproductive behaviour in male parr of brown trout (Salmo trutta). *Aquatic Toxicology* 2007; 81: 1-9.

Jaiswal, A., Parihar, V. K., Sudheer, K. M., Manjula, S.D., Krishnanand, B. R., Shanbhag, R. and Unnikrishnan, M. K.: 5-Aminosalicylic acid reverses endosulfan-induced

testicular toxicity in male rats. *Genetic Toxicology and Environmental Mutagenesis* 2005; 585: 50-59.

Jequier, A.M. Male infertility – a guide for the clinician. Oxford: Blackwell Science; 2002.

Joshi, S.C., Mathur, R. and Gulati, N.: Testicular toxicity of chlorpyrifos (an organophosphate pesticide) in albino rat. *Toxicol. Ind. Health* 2007; 23: 439-444 .

Kelce, W.R., Stone, C.R., Laws, S.C., Gray, L.E., Kemppainen, J.A. & Wilson, E.M. Persistent DDT metabolite p,p'-DDE is a potent androgen receptor antagonist. *Nature* 1995; 375: 581-585.

Khan-Dawood, F.S. & Satyawaroop, P.G. Toxic effects of chemicals and drugs on the sex accessory organs in the nonpregnant female. In: Raphael J.Witorsch (edt), Reproductive Toxicology, Second edition. Raven Press, Ltd., New York 1995. 159-173.

Kleemann, W.J., Weller, J.P., Wolf, H., Troger, H.D., Bluthgen, A. & Heeschen W. Heavy metals, chlorinated pesticides and polychlotinated biphenyls in Sudden Infant Dead Syndrome (SIDS). *International Journal Legal Medicine* 1991; 104:71-75

Klein, R.M. & Enders, G.C. Anatomy, Histology and Cell Biology 3rd Edition. McGraw-Hill Medical. New York 2007; 618p

Kumar, R., Uppal, R.P. Effect of malathion on estrous cycle and reproductive performance of rats. *J Environ Biol* 1986; 7:35-39.

Lahiri, P., Chakravarty, S., Mondal, A. & Sircar, S. Effect of lindaneon cytology and cytochemistry of exfoliated vaginal cells. *Exp Clin Endocrinol* 1985; 85:303-308.

Latchoumycandane, C. & Mathur, P. P. Effect of methoxychlor on the antioxidant system in mitochondrial and microsome-rich fractions of rat testis. *Toxicology* 2002; 176: 67-75.

Latchoumycandane, C., Chitra, K.C. & Mathur, P.P. The effect of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) induces oxidative stress in the epididymis and epididymal sperm of adult rats. *Arch. Toxicol*. 2003; 77: 280–284.

Lebel, G., Dodin, S., Ayotte, P., Marcoux, S., Ferron, L.A. & Dewailly, E. Organochlorine exposure and the risk of endometriosis. *Fertil Steril* 1998; 69:221-228.

Lenselink, D.R., Midtling, J.E. & Kolesari, G.L. Teratogenesis associated with oxydemeton-methyl in the stage 12 chick embryo. *Teratology* 1993; 48, 207–211.

Lerda, D. & Rizzi, R.: Study of reproductive function in persons occupationally exposed to 2,4- dichlorophenoxyacetic acid (2,4-D). *Mutat. Res*. 1991; 262:47–50.

Lindenau, A., Fischer, B., Seiler, P., Beier, H.M. Effects of persistent chlorinated hydrocarbons on reproductive tissues in female rabbits. *Hum Reprod* 1994; 9:772-780.

Makoto, E. & Akira, H. Development and reproductive toxicity of tributyltin and its metabolite, dibutylin, in rats. *Congenital Anomalies* 2000; 40: 108-120.

Makoto, E. & Emiko, M. Suppression of uterine decidualization correlated with reduction in serum progesterone levels as a preimplantation embryonic loss induced by diphenyltin in rats. *Reproductive toxicology* 2002; 16: 309-317.

Martin, S., Harlow, S.D. and Sowers, M.F.: DDT metabolite and androgens in African American farmers. *Epidemiology* 2002; 13: 454-458.

Martinez, E.M .& Swartz, W.J. Effects of methoxychlor on the reproductive system of the adult female mouse. Gross and histologic observations. *Reprod Toxicol* 1991; 5:139-147.

Mattison, D.R. The mechanisms of action of reproductive toxins. *Am J Ind Med* 1983; 4: 65_79.

Meeker, J.D., Barr, D.B. & Hauser, R. Human semen quality and sperm DNA damage in relation to urinary metabolites of pyrethroid insecticides. *Hum Reprod* 2008; 23(8):1932-1940.

Meeker, J.D.& Barr, D.B,, Hauser R. Pyrethroid insecticide metabolites are associated with serum hormone levels in adult men. *Reproductive Toxicology*. 2009; 27: 155-160.

Mehmet, G., Baha, O., Hilmi, D., Gulnur, T., Seren, G. G., Irfan, A., Tamer, M. Fallopian damage induced by organophosphate insecticide methyl parathion and protective effect of vitamins E and C on ultrastructural changes in rats. *Toxicology and Industrial Health* 2007; 23: 429-438.

Meltem, U., Yusuf, K., Kerem, D., Suna, K., Ayse, O. and Fatma, B.: Acute, subacute and subchronic administration of methyl parathion-induced testicular damage in male rats and protective role of vitamins C and E. *Pesticide Biochemistry and Physiology* 2007; 87:115-122.

Metzler, M., editor. The handbook of environmental chemistry. Berlin: Springer-Verlag; 2002.

Michal, F., Kenneth, M. G., Negro-Vilar A, and Skakkebæk M. Impact of the environment on reproductive health: executive summary. *Environment Health Perspective* 1993: 101(2): 159-167.

Moline, J.M., Golden, A.L., Bar-Chama, N., Smith, E., Rauch, M.E., Chapin, R.E., Perreault, S.D., Schrader, S.M., Suk, W.A. & Landrigan, P.J. Exposure to hazardous substances and male reproductive health: A research framework. *Environmental Health Perspectives* 2000; 108(9): 803-813.

Moreira, J.C., Wolff, M. Dietary and reproductive determinants of plasma organochlorine levels in pregnant women in Rio de Janeiro. *Environ Res* 2003; 91:143-50.

Morgan, C., & Ashraf, M. Reproductive Toxicity Evaluation of Pestban Insecticide Exposure in Male and Female Rats *Toxicological Research* 2008; 24(2): 137-150.

Muller, W.F., Hobson, W., Fuller, G.B., Knauf, W., Coulston F., Korte, F. Endocrine effects of chlorinated hydrocarbons in rhesus monkeys. *Ecotoxicol. Environ. Saf.* 1978; 2:161-172.

Narayana, K., Narayan, P. & D'Souza, U.J. Is our drinking water a slow poison? *Indian J. Med. Sci.* 2004; 58:528-530.

Narayana, K., Prashanthi, N., Nayanatara, A., Kumar, S.G., Kumar, H.H.C., Bairy, K.L., & D'Souza, U.J.A. A broad-spectrum organophosphate pesticide *O,O*-dimethyl *O*-4-nitrophenyl phosphorothioate (methyl parathion) adversely affects the structure and function of male accessory reproductive organs in the rat. *Environmental Toxicology and Pharmacology* 2006; 22: 315–324.

Ngoula, F., Watcho, P., Bouseko, T. S., Kenfack, A., Tchoumboué. J., Kamtchouing, P. Effects of propoxur on the reproductive system of male rats. *Afr J Reprod Health*. 2007; 11 (1):125-32.

Ngoula, F., Watcho, P., Dongmo, M-C., Kenfack, A., Kamtchouing, P., Tchoumboué. J. Effects of pirimiphos-methyl (an organophosphate insecticide) on the fertility of adult male rats. *Afr Health Sci.* 2007; 7 (1):3-9.

Ngoula, F., Tchoumboue, J., Watcho, P., Kenfack, A., Defang Fualefac, H., Manga Nzouck, J., Kamtchouing, P. Effects of dimethoate (organophosphate insecticide) on the

reproductive system and fertility of adult male rats (*Rattus norvegicus*). Presented at the international Conference for Toxicological Sciences. 31st may – 03rd June 2011, University of Buéa-Cameroon. THEME: "Searching for African Solutions to Human and Environmental Toxicological Challenge".

Nidhi, M., Pandey, G. & Jain, G.C. Pesticides: A Review of the male reproductive toxicity. *Journal of Herbal Medicine And Toxicology* 2010; 4 (1): 1-8.

Niederberger, C. Exposure to persistent organochlorine pollutants associates with human sperm Y:X chromosome ratio. *Hum Reprod* 2005; 20: 1903-1909.

Nurminen, T., Rantala, K., Kurppa, K. & Holmberg, P.C. Agricultural work during pregnancy and selected structural malformations in Finland. *Epidemiology* 1995; 6: 63-65.

Oakes, D.J., Webster W.S., Brown-Woodman P.D.C., and Ritchie H.E. A study of the potential for herbicide formulation containing 2,4-D and Picloram to cause male-medieted developmental toxicity in rats. *Toxicology Sciences* 2002; 68: 200-206.

Oduma, J.A., Wango, E.O., Makawiti, D.W., Einer-Jensen N. & Oduor-Okelo, D. Effects of graded doses of the pesticide heptachlor on body weight, mating success, oestrous cycle, gestation length and litter size in laboratory rats. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 1995a, 110:221-227.

Oduma, J.A., Wango, E.O., Oduor-Okelo, D., Makawiti, D.W. & Odongo. H. In vivo and in vitro effects of graded doses of the pesticide heptachlor on female sex steroid hormone production in rats. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 1995b; 111:191-196.

Ohi, M., Dalsenter, P.R., Andrade, A.J. & Nascimento, A.J. Reproductive adverse effects of fipronil in Wistar rats. Toxicol. Lett. 2004; 146:121-127.

Pages, N., Sauviat, M.P., Bouvet, S. & Goudey-Perriere, F. Reproductivetoxicity of lindane. *J Soc Biol* 2002; 196:325-338.

Palladino, M.A. Male reproductive system. In *Biology* Vol 3, Robinson R.(Ed). Macmillan Reference USA, New York, Thomson Gale 2002; 56-59pp.

Pant, N., Prasad, A.K., Srivastava, S.C., Shankar, R. and Srivastava, S.P.: Effect of oral administration of carbofuran on male reproductive system of rat. *Hum. Exp. Toxicol*. 1995; 14: 889-894.

Pesando, D., Robert, S., Huitorel, P., Gutknecht, E., Pereira, L., Girard, J-P., Ciapa, B. Effects of methoxychlor, dieldrin and lindane on sea urchin fertilization and early development. *Aquatic Toxicology*, 2004; 66: 225–239.

Pesticides Action Network (PAN) Belgium. Pesticides a usage domestique risques pour la sante. 1999 84 p.

Picard, A., Palavan, G., Robert, Ste-P., Pesando, D. & Ciapa, B. Effect of Organochlorine pesticides on Maturation of Starfish and Mouse Oocytes. *Toxicological Sciences*, 2003 ; 73 : 141–148.

Potashnik, G. & Abeliovich, D. Chromosomal analysis and health status of children conceived to men during or following dibromochloropropaneinduced spermatogenic suppression. *Andrologia* 1985; 17:291-6.

Prasad, A.K., Pant, N., Srivastava, S.C., Kumar, R. & Srivastava, S.P.: Effect of oral administration of carbofuran on male reproductive system of rat. *Hum. Exp. Toxicol*. 1995; 14: 484-491.

Presibella, K.M., Kita, D.H., Carneiro, C.B., Andrade, A.J., Dalsenter, P.R.. Reproductive evaluation of two pesticides combined (deltamethrin and endosulfan) in female rats. *Reprod Toxicol*. 2005; 20(1):95-101.

Ramadan, A.A.; Bakry, N.M.; Marei, A.S.M.; Eldefrawi, A.T. & Eldefrawi, M.E. Actions of pyrethroids on the peripheral benzodiazepine receptor. *Pesticide Biochemistry and Physiology* 1988; 32: 106-113.

Rani, A., Sahai, A., Srivastava, A.K. & Rani, A. Carbaryl induced histopathological changes in the testis of albino rats. *Journal of the Anatomical Society of India* 2007; 56: 4-6.

Rani, B.E., Krishnakumari, M.K. Prenatal toxicity of heptachlor in albino rats. *Pharmacol Toxicol* 1995; 76:112-114.

Rao, M., Narayana, K., Benjamin, S. & Bairy, K.L. L-ascorbic acid ameliorates postnatal endosulfan induced testicular damage in rats. *Indian J. Physiol. Pharmacol.* 2005; 49(3): 331-336.

Ray A, Chatterjee S, Ghosh S, Kabir SN, Pakrashi A, Deb C. Suppressive effect of quinalphos on the activity of accessory sex glands and plasma concentrations of gonadotrophins and testosterone in rats. *Arch Environ Contam Toxicol.* 1991; 21(3):383-387.

Ribeiro, G. Os pesticidas como disruptores endócrinos nos peixes. http://www.fmv.utl.pt/stf/ sem0001/G04.htm.

Rochelle, W.Tyl. Developmental toxicity in toxicologic research and testing. In Bryan Ballantyne (edt): Perspectives in basic and applied toxicology wright. Butterworth and Co (Publishers), London, Baston, Durban, Singapore, Sydney, Toronto, Wellington. 1988; pp 206-239.

Rowland, A.S. Pesticides and birth defects. *Epidemiology* 1995; 6 (1): 6-7.

Rupa, D.S., Reddy, P.P. and Reddi, O.S.: Reproductive performance in population exposed to pesticides in cotton fields in India. *Environ. Res.* 1991; 55: 123-128.

Sadler, T.W. Langman's Medical Embryology 10th Edition 2006; 358p

Sancewicz-Pach, K., Groszek, B., Pach, D., Klys, M. Acute pesticides poisonings in pregnant women, Przegl. Lek 54 1997; 10: 741-744.

Sang-Hee, J., Byung-Yong, K., Hwan-Goo, K., Hyun-Ok, K. & Joon-Hyoung, C. Effect of chlorpyrifos-methyl on steroid and thyroid hormones in rat F0- and F1-generations. *Toxicology* 220 (2006) 189–202.

Santamarta, J. Por um futuro sem contaminantes orgânicos persistentes. *Agroecologia e Desenvolvimento Rural Sustentável* 2001; 2:46-56.

Saradha, B., Vaithinathan, S. & Mathur, P.P. Single exposure to low dose of lindane causes transient decrease in testicular steroidogenesis in adult male Wistar rats. *Toxicology* 2008, 244: 190–197.

Saradha, B., Vaithinathan, S. & Mathur, P.P.: Lindane induces testicular apoptosis in adult Wistar rats through the involvement of Fas-FasL and mitochondria-dependent pathways. *Toxicology* 2009; 255:131-139.

Sayým, F. Histopathological effects of dimethoate on testes of rats. *Bulletin of Environmental Contamination and Toxicology* 2007; 78: 479-484.

Scheib, D. Effects and role of estrogens in avian gonadal differentiation. *Differentiation*, 1983; 23 (suppl): 87-92.

Shalaby, M.A., El Zorba, H.Y. & Ziada, R. M. Reproductive toxicity of methomyl insecticide in male rats and protective effect of folic acid. *Food and Chemical Toxicology* 2010 ; 48: 3221–3226.

Sharpe, R.M. Hormones and testis development and the possible adverse effects of environmental chemicals. *Toxicology Letters* 2001; 120: 221–232.

Sharpe, R.M., Atanassova, N., McKinnell, C., Parte, P., Turner, K.J., Fisher, J.S., Kerr, J.B., Groome, N.P., Macpherson, S., Millar, M.R. & Saunders, P.T. Abnormalities in functional development of the Sertoli cells in rats treated neonatally with diethylstilbestrol: a possible role for estrogens in Sertoli cell development. *Biology of Reproduction* 1998; 59: 1084-1094.

Sobarzo, C. & Bustoos-Obregón E. Sperm quality in mice acutely treated with parathion. Asian *Journal of Andrology*, 2000[a]; 2: 147-150.

Sobrazo, C. & Bustos-Obregon E. Acute effect of parathion on the seminiferous epithelium of immature mice. *Revista Chilena de Anatomia* 2000[b]; 18(1): 6-68.

Spencer, F. and Z. Berhane. Uterine and fetal characteristics in rats following a post-implantational exposure to permethrin. *Bull. Environ. Contam. Toxicol.* 1982; 29:84-88.

Suresh, C.J., Reena, Ma., Anita, G. &Tripta, S. Influence of methyl parathion on reproductive parameters in male rats. *Environmental Toxicology and Pharmacology* 2003 ; 14: 91-98.

Swartz, W.J., Mall, G.M. Chlordecone-induced follicular toxicity inmouse ovaries. *Reprod Toxicol* 1989; 3:203-206.

Talens, G. & Wooley, D. Effects of parathion administration during gestation in the rat on development of the young. *Proceedings of the Western Pharmacological Society* 1973; 16: 141–145.

Toppari, J. Male reproductive health and environmental xenoestrogens. Environ Health Perspect 1996; 104:741-803.

U.S. EPA. Data evaluation record: Developmental neurotoxicity study - rat; OPPTS 870.6300. (MRID 44501102.) Washington, D.C. 1997.

U.S. EPA. Data evaluation record-supplemental: Developmental neurotoxicity study - rat; OPPTS 870.6300. (MRID 44039002.) Washington, D.C. 1998[2]

U.S. EPA. Office of Pesticide Programs. "Tox one-liners": Cyfluthrin. Washington, D.C. 1989.

U.S. EPA. Office of Pesticide Programs. Health Effects Division. Tox oneliners: Permethrin. Washington D.C. 1997.

U.S. EPA. Office of Prevention, Pesticide and Toxic Substances. Fipronil for use on rice (Regent®, Icon®) and pets (Frontline®). HED risk assessment. Washington, D.C. 1998[1], 22. Pp.19,20,22.

Uphouse, L., Mason, G. & Hunter, V. Persistent vaginal estrus and serum hormones after chlordecone (kepone) treatment of adult female rats. *Toxicol Appl Pharmacol* 1984, 72:177-186.

Uzun, F.G., Kalender, S., Durak, D., Demir, F. & Kalender, Y. Malathion-induced testicular toxicity in male rats and the protective effect of vitamins C and E. *Food Chem. Toxicol.* 2009; 47: 1903-1908.

Vaithinathan, S., Saradha, B. & Mathur, P.P. Transient inhibitory effect of methoxychlor on testicular steroidogenesis in rat. *Arch. Toxicol.* 2008; 82: 833-839.

Van Emden, H.F. & Pealall, D.B. Beyond Silent Spring, Chapman & Hall, London, 1996; 322p

Vartiainen, T., Kartovaara, L. & Tuomisto, J. Environmental Chemichals and Changes in Sex Ratio: Analysis Over 250 Years in Finland. *Environmental Health Perspectives* 1999; Volume 107(10).

Villeneuve, D.C., Willes, R.F., Lacroix, J.B. & Phillips, W.E.J. Placental transfer of 14C-parathion administered intravenously to rats. *Toxicology and Applied Pharmacology* 1972; 21: 542–548.

Vogin, E.E., Carson, S. &Slomka, M.B. Teratology studies with dichlorvos in rabbits. *Toxicology and Applied pharmacology* 1971; 19: 377–378.

Waissmann, W. Endocrinopatologia associada ao trabalho. In: Mendes R, organizador. Patologia do trabalho. São Paulo: *Editora Atheneu*; 2003; p.1093-138.

Walsh, L.P. & Stocco D.M. The effects of roundup and dimethoate on steroidogenesis acute regulatory (Star) protein in mouse MA-10 Leydig cells. *Proceedings of the Society For the Study of Reproduction* 1998; 8-11: 182.

Walsh, L.P., McComick, C., Martin C., & Stocco D.M. Roundup inhibits Steroidogenesis by disrupting steroidogenic acute regulatory (StAR) protein expression. *Environmental Health Perspectives* 2000; 108 (8): 769-776.

Ware, G.W. & Whitacre DM. The Pesticide Book, 6th Ed. Meister Media Worldwide, Willoughby, Ohio. 2004; 496 pp.

Ware, G.W. An introduction to insecticides 3rd Edition 2001; p. 7

Weck, M.A. Female reproductive system. In *Biology* Vol2, Robinson. (Ed). Macmillan Reference USA, New York, Thomson Gale 2002; 77- 81 pp

Whorton, D., Krauss RM, Marshall S, Milby TH. Infertility in male pesticide workers. *Lancet* 1977; 2: 1259-61.

Wikibooks Producers. Human Physiology. 2007; 426p

Wyrobek, A.J., Watchmaker, G. and Gordon, L. Sperm shape abnormalities in carbaryl-exposed employees. *Environ. Health Perspect*. 1981; 40: 255-26

20

Pyrethroid Resistance in Insects: Genes, Mechanisms, and Regulation

Nannan Liu
Department of Entomology and Plant Pathology
Insect Molecular Toxicology and Physiology Program
Auburn University, Auburn,
Alabama,
USA

1. Introduction

Insecticides are the most important component in insect pest-control efforts worldwide. Pyrethroids are among the insecticides that have been most widely used for this purpose for many years because of their safe, cheap, effective and long-lasting nature (Bulter et al. 2011). However, the widespread development of insecticide resistance, especially pyrethroid resistance, and the fact that resistance to an insecticide generally confers cross-resistance to other insecticides has become an immense practical problem challenging the control of agriculturally, economically, and medically important insect pests and resulting in the rise of insect vector-borne diseases in many parts of the world (Zaim 2002; Bulter 2011). Insecticide resistance is a pre-adaptive phenomenon, in the sense that prior to an organism's (e.g., a mosquito's) exposure to insecticides, rare individuals (resistant individuals) carrying one or more possible resistance genes (or an altered [varied] genome) already exist, allowing the organism to survive exposure to the insecticides (WHO 1957). A large number of studies have indicated that multiple resistance mechanisms or genes are involved in the development of insecticide resistance in many insect species, including mosquitoes and house flies (Raymond et al. 1989; Hemingway et al. 2002; 2004; Liu and Scott 1995; 1996; 1997; 1998; Liu and Yue 2000; 2001; Ranson et al. 2002; Liu et al. 2005; Vontas et al. 2005; Xu et al. 2005; Liu et al. 2007; Zhu and Liu 2008; Zhu et al. 2008a; b; Liu et al. 2011). Characterization of the molecular mechanisms and genes involved in insecticide resistance has therefore been fundamental in understanding the development of resistance and in practical applications such as designing novel strategies to prevent or minimize the spread and evolution of resistance development and control insect pests.

Three major mechanisms are involved in insecticide resistance: (1) increased metabolic detoxification of insecticides; (2) decreased sensitivity of the target proteins on which an insecticide acts, known as target site insensitivity; and (3) decreased cuticular penetration/or increased sequestration/storage. Gene overexpression, amplification, and structural mutations have been linked to insecticide resistance mechanisms in some insects, while transcriptional overexpression of genes in resistant insects appears to be a common determining event in the evolution of resistance in insects generally. Regulatory interaction

among different mechanisms and/or genes in resistance offers a tantalizing hint regarding precisely how these high levels of resistance in insects may be developed. There is, however, an urgent need to improve our understanding of the mechanisms governing resistance development, which is essential if we are to develop new and more effective strategies to circumvent and/or delay resistance development, control resistant insect pests, and reduce the prevalence of insect vector-borne diseases. In this review, I will focus on the genes and mechanisms, along with their interactions and regulation, that lead to the development of insecticide resistance and discuss the potential impact of a better understanding of resistance mechanisms on the basic and practical aspects of research into how mosquitoes and house flies develop a resistance to insecticides.

2. Metabolic detoxification-mediated resistance

The mechanism of increased detoxification contributes to a decrease in the effective dose of insecticides available at the target site (Scott 1990; 1999; Feyereisen 1995; Pasteur 1996). The products of three gene families, cytochrome P450 monooxygenases (cytochrome P450s), hydrolases, and glutathione S-transferases (GSTs), are primarily implicated in the detoxification of insecticides. In the mosquito, for example, the detoxification of insecticides in the mosquito involves cytochrome P450s (P450s), esterases, and GSTs (Feyereisen 2005; Ranson and Hemingway 2005; Oakeshott et al. 2005). Insect cytochrome P450s in particular are known to play an important role in detoxifying insecticides (Scott 1999; Feyereisen 2005). Transcriptional up-regulation of P450s results in an increase in P450 proteins and P450 activities, which, in turn, trigger the development of insecticide resistance. Esterases are a group of heterogeneous enzymes present in most organisms. Although an increase in the DNA amplification of esterases has been associated with esterase-mediated insecticide resistance (Hemingway and Karunaratne 1998; Small and Hemingway 2000), non-elevated esterase levels have also been identified in mosquitoes exhibiting insecticide resistance (Whyard et al. 1995). GSTs are soluble dimeric proteins involved in the metabolism, detoxification, and excretion of a large number of endogenous and exogenous compounds (Ranson and Hemingway 2005). The up-regulation of GST genes has been identified in the mosquito *Anopheles gambiae*, which is resistant to pyrethroids (Ortelli et al. 2003). In other insect species such as the diamondback moth and the rice brown plant hopper, up-regulation of GST genes is also known to be involved in insecticide resistance (Vontas et al. 2005). Even though the detoxification gene families involved in insecticide resistance are known, however, little is known about how many genes in each family are directly involved in resistance in a resistant insect. The molecular mechanisms involved in insecticide resistance in general, and the regulation of up-regulated P450 genes, GSTs, and esterases in particular, are not yet clear.

3. P450-mediated pyrethroid resistance

Cytochrome P450s constitute one of the largest gene superfamilies in all living organisms, including mammals, fish, arthropods, fungi, plants, and bacteria, and they are known to perform a large number of highly diverse physiological and biochemical functions. In insects, more than 1700 P450s have so far been identified (Nelson 2009, http://drnelson.uthsc.edu/CytochromeP450.html). Cytochrome P450s have long been of particular interest because they are critical for the detoxification and/or activation of

xenobiotics such as drugs, pesticides, plant toxins, chemical carcinogens and mutagens. They are also involved in metabolizing endogenous compounds such as hormones, fatty acids, and steroids. Insect cytochrome P450s are known to play an important role in the detoxification and/or activation of xenobiotics such as insecticides (Scott 1999; Feyereisen, 2005) and plant toxins (Berenbaum 1991; Schuler 1996), leading to the development of resistance to insecticides (Carino et al. 1994; Liu and Scott 1997; 1998; Feyereisen 2005; Zhu et al. 2008a; Komagata et al. 2010; Liu et al. 2011) and tolerance to plant toxins (Li et al. 2002; Wen et al. 2003). Basal and up-regulation of P450 gene expression can significantly affect the disposition of xenobiotics or endogenous compounds in the tissues of organisms and thus alter their pharmacological/toxicological effects (Pavek and Dvorak 2008). Increased P450-mediated detoxification has been found in many insect species associated with enhanced metabolic detoxification of insecticides, as evidenced by the increased levels of P450 proteins and P450 activity that result from constitutively transcriptional overexpression of P450 genes in insecticide resistant insects. In addition, some insect P450 genes can be induced by exogenous compounds. Recently, our group has identified that multiple P450 genes are overexpressed in insecticide resistant house flies *Musca domestica* and mosquitoes *Culex quinquefasciatus* through both constitutive overexpression and induction by pyrethroids ((Zhu and Liu 2008; Zhu et al. 2008a; b; Liu et al. 2011), suggesting that constitutive overexpression and induction are key factors for increased levels of detoxification of insecticides and insecticide resistance. Nevertheless, although their importance in insect physiology and toxicology is widely recognized, there are enormous gaps in our knowledge of insect P450s. In particular, their precise role in the regulation processes that govern the evolution of insecticide resistance, which typically requires the interaction of multiple genes, has not yet been determined. As more P450 sequences become available, our understanding of the roles of P450s in physiological and toxicological processes should be improved.

The initial characterization of the importance of metabolic detoxification in insecticide resistance has primarily been documented in many insect species on the basis of synergistic studies. These studies have revealed that resistance to insecticides is decreased by piperonyl butoxide (PBO), the inhibitor of cytochrome P450 monooxygenases. In a series of studies in our laboratory designed to investigate how pyrethroid resistance develops, a house fly strain ALHF^{G0} and a mosquito strain HAmCq^{G0} were collected from sites in Alabama and further selected with permethrin for 6 and 8 generations, respectively, generating the strains of ALHF and HAmCq^{G8} (Liu and Yue 2000; 2001; Liu et al. 2004a; Xu et al. 2006a; Li et al. 2010). Both permethrin selected house fly and mosquito strains achieved very high levels of resistance compared with their parental strains. Synergism studies found that permethrin resistance in ALHF house flies and HAmCq mosquitoes (both- the parental strains and their selected offspring) was largely suppressed by piperonyl butoxide (PBO), indicating that P450 monooxygenase-mediated detoxification may be one of the major mechanisms involved in the development of pyrethroid resistance in these insect species (Liu and Yue 2000; Xu et al. 2005).

4. Constitutive overexpression of P450 genes in pyrethroid resistant house flies and mosquitoes

The increase in the levels of P450 proteins and P450 activities that results from the constitutive overexpression of P450 genes in insecticide resistant insects has been clearly

implicated in the development of resistance to insecticides. Based on this critical finding, our group has examined the expression profiles of P450 genes from both house flies and mosquito *Cx. quinquefasciatus*, comparing susceptible and resistant populations and the field parental population and their permethrin selected offspring. The differential expression patterns of all eight P450 genes, *CYP4G13v1*, *CYP4D4v2*, *CYP4G2*, *CYP6A5v2*, *CYP6A36*, *CYP6A37*, *CYP6A38*, and *CYP28B1*, were characterized for resistant ALHF and susceptible CS house fly strains and for different types of tissues using Northern blot analyses and quantitative real-time PCR (qRT-PCR) (Zhu and Liu 2008; Zhu et al. 2008a; b). No significant difference in the expression of *CYP4G13v1*, *CYP4D4v2*, *CYP4G2*, *CYP6A37*, *CYP6A38*, and *CYP28B1* between resistant ALHF and susceptible CS and aabys flies was observed, but *CYP6A5v2* and *CYP6A36* showed significant constitutive overexpression in the resistant ALHF strain. Four cytochrome P450 cDNAs, *CYP6AA7*, *CYP9J40*, *CYP9J34*, and *CYP9M10*, were also isolated from mosquitoes, *Cx. quinquefasciatus* (Liu et al. 2011) and their expression was compared for three different mosquito populations bearing different resistance phenotypes, ranging from susceptible (S-Lab), through intermediate ($HAmCq^{G0}$, the field parental population) to highly resistant ($HAmCq^{G8}$, the 8th generation of permethrin selected offspring of $HAmCq^{G0}$). A strong correlation was found for all P450 gene expression with the levels of resistance and following permethrin selection at the larval stage of mosquitoes, with the highest expression levels identified in $HAmCq^{G8}$, suggesting the importance of *CYP6AA7*, *CYP9J40*, *CYP9J34*, and *CYP9M10* in the permethrin resistance of larva mosquitoes.

Another important feature of insect P450 genes is that they may vary with regard to the tissues where they are expressed in response to physiological and environmental stimulators. In insects, the midgut and fat body tissues are generally considered to be the primary detoxification organs where most insect detoxification P450s are expressed (Hodgson 1985; Scott et al. 1998). The tissue specific expression of two P450 genes, *CYP6A36* and *CYP6A5v2*, from house flies and *CYP6AA7* from *Cx. quinquefasciatus* was examined because these genes overexpressed not only in resistant insects but also in adult stages. Although the expression of *CYP6A5v2* was observed to be significantly higher in the abdominal tissue in both the susceptible CS and aabys flies and the resistant ALHF flies compared with their head+thorax tissues, this overexpression was far greater in both sets of tissues for resistant ALHF flies than in the tissues of susceptible flies (Zhu and Liu 2008). The expression of *CYP6A36* was not significantly different between the head+thorax and abdomen tissues of the CS strain; lower in the head+thorax tissue than in the abdomen tissue of ALHF; and significantly higher in both tissues of the ALHF strain than in the CS strain (Zhu et al. 2008a). Significant overexpression of *CYP6AA7* in resistant $HAmCq^{G8}$ mosquitoes was found in all three types of tissues, head, thorax, and abdomen (Liu et al. 2011) compared with susceptible S-Lab mosquitoes, with the highest levels expressed being in the abdomen tissue. As midgut and most fat body components are located in the abdomen of insects and are known to be of primary importance in detoxification-related functions, the overexpression of the 3 P450 genes, *CYP6A5v2* and *CYP6A36* in house flies and CYP6AA7 in mosquitoes, specifically in the abdomen tissues of resistant insect populations, suggests the importance of these genes in increasing the metabolic detoxification of insecticides in house flies and mosquitoes. However, because midgut and fat body tissues are not exclusively found in the abdomen, further dissection of detoxification-related tissues (such as midgut and fat

body) is needed to pinpoint the precise location for the overexpression of these resistance related genes.

5. Induction of P450 genes in response to permethrin exposure in house flies and mosquitoes

Another characteristic of some insect P450 genes is that their expression can be induced by exogenous and endogenous compounds (Feyereisen 2005), a phenomenon known as induction. It has been suggested that many chemical inducers may act as substrates for the P450s that they induce and that the induction of the P450s by the substrates will, in turn, reduce the effects of the substrates by enhancing substrate metabolism (Okey 1990). The involvement of the induction of P450s and their activities in the adaptation of insects to their environment and the development of insecticide resistance was proposed by Terriere (1983; 1984), who argued that while all insects probably possessed some capacity to detoxify insecticides and xenobiotics, the degree to which they can metabolize and detoxify these highly toxic chemicals was of considerable importance to their survival in a chemically unfriendly environment (Terriere 1984). Both constitutively increased expression (overexpression) and induction of P450s are thought to be responsible for increased levels of detoxification of insecticides. Multiple P450 genes induced in insects in response to host plant allelochemicals or secondary products have been extensively studied and are fairly well documented in terms of their function in the adaptation of insects in "animal-plant warfare" (Gonzalez and Nebert 1990) and in the co-evolution of insects and plants (Li et al. 2002). In contrast, P450 gene induction in response to insecticide resistance is less well understood.

The induction profiles of P450 genes in house flies and mosquitoes have been characterized in our laboratory. Dose range, time course, and P450 gene induction assays on *Cx. quinquefasciatus* showed a clear time- and dose -dependent response of mosquito P450s to permethrin (Liu et al. 2011). No significant induction was detected in the expression of *CYP6AA7*, *CYP9J34*, and *CYP9M10* in susceptible S-Lab mosquitoes, but various levels of induction were identified in the field parental mosquitoes $HAmCq^{G0}$ and their permethrin selected offspring $HAmCq^{G8}$. No significant expression of *CYP6AA7* was detected in $HAmCq^{G0}$ that had been treated with either acetone alone (control) or with permethrin at 24 h. However, in the $HAmCq^{G8}$ strain an initial induction of *CYP6AA7* (~1.5-fold) was found in mosquitoes that had been treated with a LC_{10} concentration of permethrin and a marked induction (~4.5-fold) in those treated with the permethrin at a concentration of LC_{50}. Levels of *CYP9J34* RNA in $HAmCq^{G0}$ were readily induced by a LC_{10} permethrin concentration, reaching a maximum (~1.7-fold) for a LC_{50} permethrin concentration, with no further significant induction up to LC_{90}. The induction of *CYP9J34* was even more evident in the $HAmCq^{G8}$ strain than in their parental $HAmCq^{G0}$, with an induction peak of ~2.7-fold at a permethrin concentration of LC_{50}. A similar induction pattern was found for *CYP9M10* in $HAmCq^{G0}$ and $HAmCq^{G8}$.

Comparable results for the time- and dose -dependent induction by permethrin on P450 gene expression have also been identified in house flies (Zhu et al. 2008b). The expression patterns of eight P450 genes, *CYP4G13v1*, *CYP4D4v2*, *CYP4G2*, *CYP6A5v2*, *CYP6A36*, *CYP6A37*, *CYP6A38*, and *CYP28B1* in response to permethrin treatment in resistant ALHF and susceptible CS and aabys house flies were characterization by treating 2-day old adult house flies with permethrin. Three P450 genes, *CYP4D4v2*, *CYP4G2*, and *CYP6A38*, were co-

up-regulated by permethrin treatment in permethrin resistant ALHF house flies with a LD_{50} dose and a 24 h time interval (Zhu et al. 2008b). No significant induction in the expression of these three P450 genes was found in susceptible house flies that had either been treated with acetone alone or with permethrin solution in acetone compared with untreated house flies. Similarly, no significant induction was obtained in acetone treated ALHF house flies compared with their untreated counterparts. However, all three of these genes were induced at various levels in permethrin treated ALHF house flies compared with untreated or acetone treated flies. There was a marked induction of *CYP4D4v2* and *CYP6A38* mRNA in permethrin treated ALHF house flies, whereas a low level of induction for *CYP4G2* was detected in the permethrin treated ALHF house flies (Zhu et al. 2008b).

The significant induction of the P450 genes only in the field resistant and/or permethrin selected highly resistant strains of both house flies and mosquitoes strongly suggests the importance of P450 genes in the resistant insects, particularly with regard to their response to permethrin treatment. Taken together, these results indicate that multiple P450 genes are up-regulated in insecticide resistant insects through both constitutive overexpression and induction mechanisms, thus increasing the overall expression levels of P450 genes.

6. Sodium channel mutation-mediated target-site insensitivity in pyrethroid resistance

The mechanism of decreased target site sensitivity contributes to the ineffective binding of a given dose of inscticides (Scott 1990; 1999; Feyereisen 1995; Pasteur and Raymond 1996). Target-site insensitivity results from the structural modification or mutation (point mutation) of the target proteins that the insecticides act upon. Point mutations in the target protein cause a reduction in the nervous system's response to insecticides (or a reduction in the binding affinity of the protein to insecticides) (Narahashi 1988) which, in turn, enhances the insect's resistance to insecticides. Insecticides such as DDT and pyrethroids specifically target the sodium channels in the nervous system (Narahashi 1988; 1996).

Pyrethroids and DDT deliver their toxic, insecticidal effects primarily by binding onto the sodium channel, altering its gating properties and keeping the sodium channel open for unusually longer time, thereby causing a prolonged flow of sodium current. This prolonged sodium current elevates and prolongs the depolarizing phase of the action potential of the neuron membrane, which initiates repetitive discharges and prevents the repolarization phase of action potentials (Narahashi 1988). However, modifications in the sodium channel structure (i.e., point mutation or substitution, resulting from single nucleotide polymorphisms [SNP]) cause insensitivity to DDT and pyrethroids in the sodium channels of the insect's nervous system, via a reduction in or an elimination of the binding affinity of the insecticides to proteins (Narahashi 1988), thus diminishing the toxic effects of the insecticides and resulting in insecticide resistance (Soderlund 2005; Dong 2007). Reduced target-site sensitivity of sodium channels is known to be one of the major mechanisms involved in pyrethroid resistance and is referred to as knockdown resistance (kdr) (Soderlund and Knipple 2003).

As part of our effort to characterize all the mutations in an entire mosquito sodium channel that are involved in pyrethroid resistance, we first cloned and sequenced the full-length of the sodium channel cDNA for *Cx. quinquefasciatus* and identified, for the first time, both nonsynonymous and synonymous mutations, including the L-to-F *kdr* mutation, that are

co-present in the sodium channel of individual mosquitoes (Xu et al. 2011). With the full length of the sodium channel cDNA clones and a large number of mutations identified, we are now in an excellent position to fully characterize the key mutations contributing to insecticide resistance in an entire mosquito sodium channel, and we plan to investigate the effects of these key mutations and mutation combinations on the sensitivity of the mosquito sodium channel to pyrethroids.

To shed light on the connection between sodium channel mutations and pyrethroid resistance, Xu et al. (2006a; b) compared genomic DNA and RNA expression levels within the same individuals for mosquitoes *Cx. quinquefasciatu*s, house flies *M. domestica*, and German cockroaches *Blattella germanica* bearing different resistant phenotypes ranging from susceptible to highly resistant. In these studies, no correlation for the L-to-F allele at the genomic DNA level with either level of susceptibility or resistance to insecticide was identified in any of the three insect species examined. However, a strong correlation between the L-to-F allele expression and levels of insecticide resistance and susceptibility was observed. These findings offer a completely new approach to exploring how these target site genotypes and their insensitivity-mediated resistance phenotypes are coupled.

Building on the above research, an in-depth investigation of the genomic organization and allelic expression at the L-to-F site of the sodium channel gene in *Cx. quinquefasciatus* has been conducted (Xu et al. 2011). Multiple copies of the sodium channel gene were identified in the mosquito *Cx. quinquefasciatus* by Southern blot analysis and polymerase chain reaction (PCR) analysis (Xu et al. 2011). Two genomic DNA fragments of the mosquito sodium channel gene (509 bp and 181 bp) were detected by a single PCR primer pair. Sequence analysis indicated the lack of an intron sequence in the 181 bp sodium channel fragment. Single nucleotide polymorphism (SNP) analysis revealed a strong correlation among the frequencies of L-to-F allelic (T) expression at the RNA level, the frequencies and resistance allele (T) at the L-to-F site of the 509 bp genomic DNA fragment (which did include an intron sequence), and the levels of insecticide resistance. This study, for the first time, not only revealed that multiple copies of the sodium channel gene are present in the *Culex* mosquito genome but also suggested that the copy containing the intron sequence may be a functional copy of the sodium channel gene in *Culex* mosquitoes. Further investigation of the role of these multiple copies of the sodium channel gene in the genome of mosquitoes will provide a more comprehensive picture of the mechanisms involved in the development of sodium channel mediated pyrethroid resistance in mosquitoes.

7. Multiple gene interactions in pyrethroid resistance

Although it is possible that an individual mechanism acting alone could confer resistance, there is a considerable body of evidence indicating that an interaction involving multiple resistance mechanisms or genes is responsible for high levels of insecticide resistance. Results from both our studies and those of many other research groups suggest that the interaction of multiple insecticide resistance mechanisms or genes is likely to be responsible for the development of insecticide resistance. For example, multiple genes have been found to be up-regulated in insecticide resistant HAmCq mosquitoes (Liu et al. 2007). Apart from two of the P450 genes, most of the up-regulated genes had not previously been reported in the literature on insecticide resistance. Two of the overexpressed genes are rhodopsin and arrestin, both of which play a crucial role in signal transduction systems. Rhodopsin is an archetypal class A G-protein-coupled receptor (GPCR) (Filipek et al. 2003). GPCRs act as

transducers for a range of different sensory, chemotactic, hormonal, and neuronal signals, and are involved in many essential physiological functions of organisms. Arrestins are members of a gene family of regulatory proteins that, along with G-protein-coupled receptor kinases (GRKs) and other co-factors, regulate the signaling and trafficking of G-protein-coupled receptors by virtue of their preferential binding to the phosphorylated active form of the receptor. Up-regulation of these two genes in resistant mosquitoes may indicate that the neuronal signaling is affected in resistant mosquitoes. The resistance-specific overexpression of arrestins and rhodopsin in resistant mosquitoes (Liu et al. 2007) highlights the functional importance of the signal transduction system in the regulation of insecticide resistance. Similarly, Vontas's group reported multiple genes (including a P450 gene, *CYP314A1*) to be up-regulated in DDT-resistant *Anopheles gambiae* mosquitoes (Vontas et al. 2006). While it is unknown whether and how these up-regulated genes are associated with insecticide resistance, the results of these two studies not only indicate a common phenomenon of insecticide resistance conferred by multi-resistance mechanism interactions, but also offer a tantalizing hint of a regulatory relationship among different mechanisms and/or genes in resistance.

8. The *trans* and/or *cis* regulation in pyrethroid resistance

The regulation of *trans* and/or *cis* genes (factors) that account for the same resistance mechanism may provide another explanation for high levels of resistance. Many studies have demonstrated that the up-regulation of P450 and GST genes in resistant insects is regulated by *trans* and/or *cis* regulatory genes. The up-regulation of a GST gene (*GST-2*) in the mosquito *Aedes aegypti* is controlled by a *trans*-acting factor (Grant and Hammock 1992), while the up-regulation of 2 P450 genes, *CYP6A1* and *CYP6D1*, in the house fly *M. domestica* is known to be *trans*-regulated by one or more factors on autosome 2 (Carino et al. 1994; Liu and Scott 1996). The up-regulation of *CYP6A2* and *CYP6A8* in the fruit fly *Drosophila melanogaster* is transcriptionally regulated by *trans*-regulatory factors (Maitra 2000).

Our group has also recently examined trans and/or cis regulation of insecticide resistance or resistance genes in house flies by characterizing autosomal interactions and contributions, both individually and in combination, and its influence on the development of pyrethroid resistance (Tian et al. 2011). Five BC_1 lines and 16 mass-cross homozygous lines were generated from crosses of the pyrethroid resistant ALHF (wild-type) and susceptible aabys (bearing recessive morphological markers on each of five autosomes) strains. The resulting homozygous lines all had combinations of autosomes that differed from those of the resistant ALHF strain. The results indicated that factors on autosome 4 are not involved in the development of resistance in house flies, while factors on autosomes 1, 2, 3 and 5 play important roles in pyrethroid resistance. The sodium channel gene mapped onto autosome 3 and multiple cytochrome P450 genes overexpressed in resistant ALHF house flies were genetically mapped on autosome 5, suggesting that sodium channel-mediated target site insensitivity and P450-mediated detoxification located on autosomes 3 and 5, respectively, are major factors related to the development of resistance in house flies. However, neither of the factors on autosome 3 or 5 alone, nor the factors from both autosomes 3 and 5 combined, could confer the high levels of resistance to pyrethroids actually observed. Strong synergistic effects on resistance are obtained when autosomes 1 and 2 interact with autosome 3 and/or 5 however, suggesting that the trans factors on autosomes 1 and 2 may interact with factors on autosomes 3 and

5 and thus play a regulatory role in the development of sodium channel insensitivity- and P450 detoxification-mediated resistance.

Taken together, the above findings not only indicate a common phenomenon of insecticide resistance conferred by multi-resistance mechanism interactions, but also offer tantalizing clues suggesting a regulatory relationship among different mechanisms and/or genes in resistance, although none of these regulatory genes has as yet been characterized.

9. Acknowledgments

The studies performed by the author's group were supported by Award Numbers R21AI076893 and R21AI090303 to N.L. from the National Institute of Allergy and Infectious Diseases, the U.S. Department of Agriculture National Research Initiative (USDA-NRI) Competitive Grants Program; AAES Hatch/Multistate Grants ALA08-045 and ALA015-1-10026 to N.L. AAES Foundation Grants; Auburn University and Auburn University Biogrant 2008-Bio-Liu-03-08; the Hatch Project ALA08-029; and the Department of Entomology and Plant Pathology, Auburn University. The author sincerely thanks all the people who have ever worked and are currently working in her laboratory for their hard work and dedication to the field of insecticide resistance research.

10. References

Berenbaum, M.R., 1991. Coumarins. In: Rosenthal, G.A., Berenbaum, M.R. (Eds.), Herbivores: Their Interaction with Secondary Plant Metabolites, Academic Press, New York, pp. 221-249.

Butler, D., 2011. Mosquitoes score in chemical war. Nature 475, 19-20.

Carino, F.A., Koener, J.F., Plapp, F.W., Jr., Feyereisen, R., 1994. Constitutive overexpression of the cytochrome P450 gene *CYP6A1* in a house fly strain with metabolic resistance t0 insecticides. Insect Biochem. Mol. Biol. 24, 411-418.

Dong, K., 2007. Insect sodium channels and insecticide resistance. Invert. Neurosc. 7, 17-30. Feyereisen, R. 1995, Toxicol. Lett., 82/83, 83-90.

Feyereisen, R., 2005. Insect cytochrome P450. In: Gilbert, L.I., Iatrou, K., Gill, S. (Eds.), Comprehensive Molecular Insect Science, vol. 4. Elsevier, Oxford, pp. 1–77.

Filipek, S., Stenkamp, R.E., Teller, D.C., Palczewski, K., 2003. G protein-coupled receptor rhodopsin: a prospectus. Annu. Rev. Physiol. 65, 851–879.

Grant, D.F., Hammock, B.D., 1992. Genetic and molecular evidence for a *trans*-acting regulatory locus controlling glutathione S-tranferase-2 expression in *Aedes aegypti*. Mol. Gen. Genet. 234, 169-176.

Gonzalez, F.J., Nebert, D.W., 1990. Evolution of the P450 gene superfamily: animal-plant "warfare", molecular drive, and human genetic differences in drug oxidation. Trends Genet. 6, 182-186.

Hemingway, J., Field, L., Vontas, J., 2002. An overview of insecticide resistance. Science 298, 96-97.

Hemingway, J., Hawkes, N.J., McCarroll, L., Ranson, H., 2004. The molecular basis of insecticide resistance in mosquitoes. Insect Biochem. Mol. Biol. 34, 653-665.

Hemingway, J., Karunaratne, S.H., 1998. Mosquito carboxylesterases: a review of the molecular biology and biochemistry of a major insecticide resistance mechanism. Med. Vet. Entomol. 12, 1-12.

Hodgson, E., 1985. Mocrosomal mono-oxygenases. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 11. Pergamon, Oxford, pp. 225-321.

Komagata, O., Kasai, S., Tomita, T., 2010. Overexpression of cytochrome P450 genes in pyrethroid-resistant *Culex quinquefasciatus*. Insect Biochem. Mol. Biol. 40, 146-152.

Li, T., Liu, N., 2010. Genetics and Inheritance of Permethrin Resistance in the Mosquito *Culex quinquefasciatus*. J. Med. Entomol. 47, 1127-1134.

Li, W., Petersen, R.A., Schuler, M.A., Berenbaum, M. R., 2002. *CYP6B* cytochrome P450 monooxygenases from *Papilio canadensis* and *Papilio glaucus*: potential contributions of sequence divergence to host plant associations. Insect Mol. Biol. 11, 543-551.

Liu, H., Cupp, E.W., Micher, K.M., Guo, A., Liu, N., 2004a. Insecticide resistance and cross-resistance in Alabama and Florida strains of *Culex quinquefasciatus*. J Med. Entom. 41, 408-413.

Liu, H., Cupp, E.W., Guo, A., Liu, N., 2004b. Insecticide resistance in Alabama and Florida mosquito strains of *Aedes albopictus*. J. Med. Entom. 41, 946-952.

Liu, N., Li, T., Reid, W.R., Yang, T., Zhang, L., 2011. Multiple cytochrome P450 genes: their constitutive overexpression and permethrin induction in insecticide resistant mosquitoes, *Culex quinquefasciatus*. *PlosOne*.

Nelson, D.R., 2009. The Cytochrome P450 Homepage. Human Genom. 4, 59-65.

Liu, N., Liu, H., Zhu, F., Zhang, L., 2007. Differential expression of genes in pyrethroid resistant and susceptible mosquitoes, *Culex quinquefasciatus*. Gene. 394, 61-68.

Liu, N., Scott, J.G., 1995. Genetics of resistance to pyrethroid insecticides in the house fly, *Musca domestica*. Pestic. Biochem. Physiol. 52, 116-124.

Liu, N., Scott, J.G., 1996. Genetic analysis of factors controlling elevated cytochrome P450, *CYP6D1*, cytochrome b5, P450 reductase and monooxyenase activities in LPR house flies, *Musca domestica*. Biochem. Genet. 34, 133-148.

Liu, N., Scott, J.G., 1997. Inheritance of *CYP6D1*-mediated pyrethroid resistance in house fly (Diptera: Muscidae). J. Econ. Entomol. 90, 1478-1481.

Liu, N., Scott, J.G., 1998. Increased transcription of *CYP6D1* causes cytochrome P450-mediated insecticide resistance in house fly. Insect Biochem. Mol. Biol. 28, 531-535.

Liu, H., Xu, Q., Zhang, L., Liu, N., 2005. Chlorpyrifos resistance in the mosquito *Culex quinquefasciatus*. J. Med. Entomol. 42, 815-820.

Liu, N., Yue, X., 2000. Insecticide resistance and cross-resistance in the house fly (Diptera: Muscidae). J. Econ. Entomol. 93, 1269-1275.

Liu, N., Yue, X., 2001. Genetics of pyrethroid resistance in a strain (ALHF) of house flies (Diptera: Muscidae). Pestic. Biochem. Physiol. 70, 151-158.

Maitra, S., Dombrowski, S.M., Basu, M., et al., 2000. Factors on the third chromosome affect the level of Cyp6a2 and Cyp6a8 expression in *Drosophila melanogaster*. Gene. 248,147-156.

Maitra, S., Dombrowski, S.M., Waters, L.C., Ganguly, R., 1996. Three second chromosome-linked clustered Cyp6 genes show differential constitutive and barbital-induced expression in DDT-resistant and susceptible strains of *Drosophila melanogaster*. Gene 180, 165–171.

Narahashi, T., 1988. Molecular and cellular approaches to neurotoxicology: past, present and future. In: Lunt, G.G. (Ed.), Neurotox '88: Molecular Basis of Drug and Pesticide Action, Elsevier, New York, pp. 563–582.

Narahashi, T., 1996. Neuronal ion channels as the target sites of insecticides. Pharmacol. Toxicol. 78, 1-14.

Oakeshott, J.G., Claudianos, C., Campbell, P.M., Newcomb, R.D., Russell, R.J., 2005. In: Gilbert, L.I., Iatrou, K., Gill, S. (Eds.), Biochemical genetics and genomics of insect esterases. Comprehensive Molecular Insect Science, vol. 5. Elsevier, Oxford, pp. 309-381.

Okey, A.B., 1990. Enzyme induction in the cytochrome P-450 system. Pharmacol. Ther. 45, 241-298.

Ortelli, F., Rossiter, L.C., Vontas, J., Ranson, H., Hemingway, J., 2003. Heterologous expression of four glutathione transferase genes genetically linked to a major insecticide-resistance locus from the malaria vector *Anopheles gambiae*. Biochem. J. 373, 957–963.

Pasteur, N., Raymond, M., 1996. Insecticide resistance genes in mosquitoes: their mutations, migration, and selection in field populations. J. Hered. 87, 444-449.

Pavek, P., Dvorak, Z., 2008. Xenobiotic-induced transcriptional regulation of xenobiotic metabolizing enzymes of the cytochrome P450 superfamily in human extrahepatic tissues. Curr. Drug Metab. 9, 129-143.

Ranson, H., Claudianos, C., Ortelli, F., Abgrall, C., Hemingway, J., Sharakhova, M.V., Unger, M.F., Collins, F.H., Feyereisen, R., 2002. Evolution of supergene families associated with insecticide resistance. Science 298, 179-181.

Ranson, H., Hemingway, J., 2005. Glutathione transferases. In: Gilbert, L.I., Iatrou, K., Gill, S. (Eds.), Comprehensive Molecular Insect Science, vol. 5. Elsevier, Oxford, pp. 383-402.

Raymond, M., Arnaouty, V.B., Sivasubramanian, N., Mouches, C., Georghiou, G.P., Pasteur, N., 1989. Amplification of various esterase B's responsible for organophosphate resistance in *Culex* mosquitoes. Biochem. Genet. 27, 417-423.

Schuler, M.A., 1996. The role of cytochrome P450 monooxygenases in plant-insect interactions. Plant Physiol. 112, 1411-1419.

Scott, J.G., 1990 Investigating mechanisms of insecticide resistance: methods, strategies and pitfalls. In: Roush R.T., Tabashnik B.E. (eds.), Pesticide Resistance in Arthropods, Chapman and Hall, New York, pp. 39-57.

Scott, J.G., 1999. Cytochromes P450 and insecticide resistance. Insect Biochem. Mol. Biol. 29, 757-777.

Scott, J.G., Liu N., Wen, Z., 1998. Insect cytochrome P450: diversity, insecticide resistance and tolerance to plant toxins. Comp. Biochem. Physiol. 121C, 147-155.

Small, G.J., Hemingway, J., 2000. Molecular characterization of the amplified carboxylesterase gene associated with organophosphorus insecticide resistance in the brown planthopper, *Nilaparvata lugens*. Insect Mol. Biol. 9, 647-653.

Soderlund, D.M., 2005. Sodium channels. In: Gilbert, L.I., Latrou K. and Gill, S.S. (Eds.), Comprehensive Molecular Insect Science, vol. 5. Elsevier BV, Oxford, pp. 1-24.

Soderlund, D.M., Knipple, D.C., 2003. The molecular biology of knockdown resistance to pyrethroid insecticides. Insect Biochem. Mol. Biol. 33, 563-577.

Terriere, L.C., 1983. Enzyme induction, gene amplification, and insect resistance to insecticides. In: Georghiou, G.P., Saito, T. (Eds.), Pest Resistance to Pesticides. Plenum Press, New York, pp. 265-297.

Terriere, L.C., 1984. Induction of detoxication enzymes in insects. Ann. Rev. Entomol. 29, 71-88.

Vontas, J., Blass, C., Koutsos, J.A.C., David, P., Kafatos, F.C., Louis, C., Hemingway, J., Christophides, G.K., Ranson, H., 2005. Gene expression in insecticide resistant and susceptible *Anopheles gambiae* strains constitutively or after insecticide exposure. Insect Mol. Biol. 14, 509-521.

Wen, Z., Pan, L., Berenbaum, M.B., Schuler, M.A., 2003. Metabolism of linear and angular furanocoumarins by *Papilio polyxenes* CYP6B1 co-expressed with NADPH cytochrome P450 reductase. Insect Biochem. Mol. Biol. 33, 937–947.

Whyard, S., Downe, A.E.R., Walker V. K., 1995. Characterization of a novel esterase conferring insecticide resistance in the mosquito *Culex tarsalis*. Arch. Insect Biochem. Physiol. 29, 329-342.

World Health Organization. 1957. Expert committee on insecticides. W.H.O. Tech. Rpt. Ser. 7th Rpt.

Xu, Q., Liu, H., Zhang, L., Liu, N., 2005. Resistance in the mosquito, *Culex quinquefasciatus* (S.), and possible mechanisms for resistance. Pest Manag. Sci 61, 1096-1102.

Xu, Q., Wang, H., Zhang, L., Liu, N., 2006a. *Kdr* allelic variation in pyrethroid resistant mosquitoes, *Culex quinquefasciatus* (S.). Biochem. Biophys. Res. Commun. 345, 774-780.

Xu, Q., Wang, H., Zhang, L., Liu, N., 2006b. Sodium channel gene expression associated with pyrethroid resistant house f lies and German cockroaches. Gene 379, 62-67.

Xu, Q., Tain, L., Zhang, L., Liu, N., 2011. Sodium channel genes and their differential genotypes at the L-to-F *kdr* locus in the mosquito *Culex quinquefasciatus*. Biochem. Biophys. Res. Com. 407, 645–649.

Zaim, M., Guillet, P., 2002. Alternative insecticides: an urgent need. Trends Parasitol. 18, 161-163.

Zhu, F., Feng, J., Zhang, L., Liu, N., 2008a. Characterization of two novel cytochrome P450 genes in insecticide-resistant house-flies. Insect Mol. Biology 17, 27-37.

Zhu, F., Li, T., Zhang, L., Liu, N., 2008b. Co-up-regulation of three P450 genes in response to permethrin exposure in permethrin resistant house flies, *Musca domestica*. BMC Physiol. 8: 18.

Zhu, F., Liu, N., 2008. Differential expression of *CYP6A5* and *CYP6A5v2* in pyrethroid-resistant house flies, *Musca domestica*. Arch. Insect Biochem. Physiol. 67, 107-119.

21

Insecticide Resistance

Sakine Ugurlu Karaağaç
Plant Protection Central Research Institute, Gayret Mah. FSM Bulvari, Yenimahalle, Ankara, Turkey

1. Introduction

Insecticide resistance is an increasing problem faced by those who need insecticides to efficiently control medical, veterinary and agricultural insect pests. In many insects, the problem extends to all major groups of insecticides. Since the first case of DDT resistance in 1947, the incidence of resistance has increased annually at an alarming rate. It has been estimated that there are at least 447 pesticide resistant arthropods species in the world today (Callaghan, 1991). Insecticide resistance has also been developed by many insects to new insecticides with different mode of action from the main four groups.

The development of resistance in the fields is influenced by various factors. These are biological, genetic and operational factors. Biological factors are generation time, number of offspring per generation and migration. Genetic factors are frequency and dominance of the resistance gene, fitness of resistance genotype and number of different resistance alleles. These factors cannot be influenced by man. However, such as treatment, persistence and insecticide chemistry, all of which may and therefore timing and dosage of insecticide application should be operational factors.

Pesticide resistance is the adaptation of pest population targeted by a pesticide resulting in decreased susceptibility to that chemical. In other words, pests develop a resistance to a chemical through natural selection: the most resistant organisms are the ones to survive and pass on their genetic traits to their offspring (PBS, 2001).

Pesticide resistance is increasing in occurrence. In the 1940s, farmers in the USA lost 7% of their crops to pests, while since the 1980s, the percentage lost has increased to 13, even though more pesticides are being used (PBS,2001). Over 500 species of pests have developed a resistance to a pesticide (Anonymous, 2007). Other sources estimate the number to be around 1000 species since 1945 (Miller, 2004).

Today, pests once major threats to human health and agriculture but that were brought under control by pesticides are on the rebound. Mosquitoes that are capable of transmitting malaria are now resistant to virtually all pesticides used against them. This problem is compounded because the organisms that cause malaria have also become resistant to drugs used to treat the disease in humans. Many populations of the corn earworm, which attacks many agricultural crops worldwide including cotton, tomatoes, tobacco and peanuts, are resistant to multiple pesticides (Berlinger, 1996).

Despite many years of research on alternative methods to control pests and diseases in crops, pesticides retain a vital role in securing global food production and this will remain the case for the foreseeable future if we wish to feed an ever growing population.

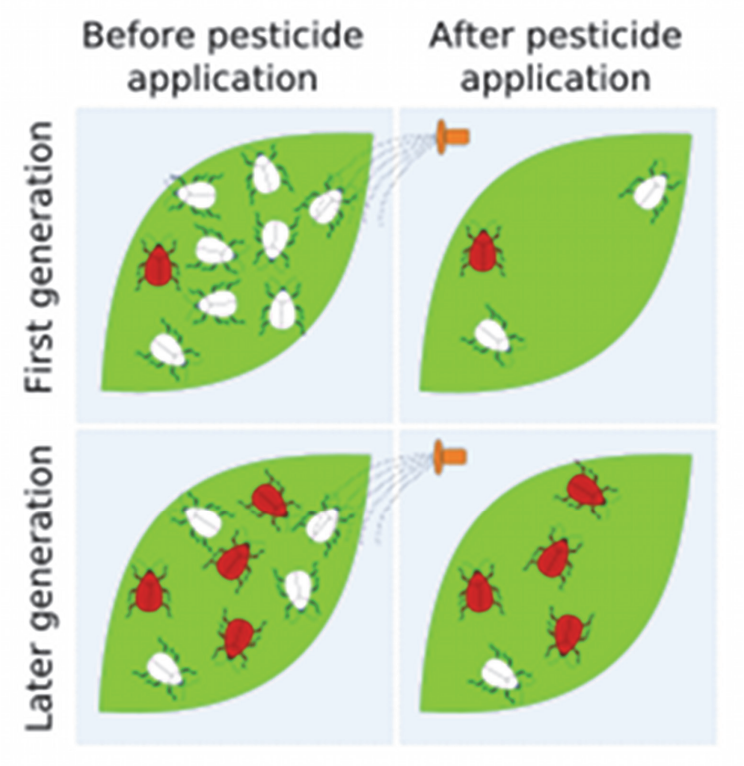


Fig. 1. Pesticide application can artificially select for resistant pests. In this figure, the first generation happens to have an insect with a heightened resistance to a pesticide (red). After pesticide application, its descendants represent a larger proportion of the population because sensitive pests (white) have been selectively killed. After repeated applications, resistant pests may comprise the majority of the population (PBS, 2001).

Insecticides are applied to reduce the number of insects that destroy crops or transmit disease in the field of agriculture, veterinary and public health. Insecticides are not always effective in controlling insects, since many populations have developed resistance to the toxic effects of the compounds. Resistance can be defined an inherited ability to tolerate a dosage of insecticide that would be lethal to the majority of individuals in a normal wild populations of the same species.

Insecticides are in common use in agriculture as well as in houseplant populations, gardens, and other living spaces in an attempt to control the invasion of a seemingly endless array of insects. Insecticides are used to keep populations under the control, but over time insects can build up a resistance to the chemicals used. This is called insecticide resistance. Insecticide resistance is apparent when a population stops responding or does not respond as well to applications of insecticides.
In recent years, many of the resistance mechanisms have been detected and resistance detection methods have been developed. These mechanisms have divided into four categories: a) increased metabolism to non-toxic products, b)decreased target site sensitivity, c)decreased rates of insecticide penetration, d) increased rates of insecticide excretion. There are different methods to determine that the mechanisms are available in any given population. We can see the structure of the resistance mechanisms from these assays.
There are several thousand species of insect in the world of particular nuisance to man, either as vectors of fatal and debilitating diseases or destroyers of crops. Insecticide resistance is an increasing problem faced by those who need insecticides to efficiently control medical, veterinary and agricultural insect pests.

2. History of insecticide resistance

In 1914 A. L. Melander reported the first case of insecticide resistance. He studied the effectiveness of lime sulphur, an inorganic insecticide, against an orchard pest, the San Jose scale (*Quadraspidiotus perniciousus*) in the state of Washington. A treatment with lime sulphur killed all scales in one week in typical orchards, but 90 percent survived after two weeks in an orchard with resistant scales. Although few cases of insecticide resistance were recorded before 1940, the number grew exponentially following widespread use of DDT and other synthetic organic insecticides (http://science.jrank.org)
Insects have evolved resistance to all types of insecticides including inorganics, DDT, cyclodienes, organophosphates, carbamates, pyrethroids, juvenile hormone analogs, chitin synthesis inhibitors, avermectins, neonicotinoids, and microbials.
In many insects, the problem extends to all major groups of insecticides. Since the first case of DDT resistance in 1947, the incidence of resistance has increased annually at an alarming rate. It has been estimated that there are at least 447 pesticide resistant arthropods species in the world today (Callaghan, 1991). Insecticide resistance has also been developed by many insects to new insecticides with different mode of action from the main four groups. For example, neoniconitoids.
Resistance occurs in thirteen orders of insects, yet more than 90 percent of the arthropod species with resistant populations are either Diptera (35 percent), Lepidoptera (15 percent), Coleoptera (14 percent), Hemiptera (in the broad sense, 14 percent), or mites (14 percent). The disproportionately high number of resistant Diptera reflects intense use of insecticides against mosquitoes that transmit disease. Agricultural pests account for 59 percent of harmful resistant species while medical and veterinary pests account for 41 percent. Many species have numerous resistant populations, each of which resists many insecticides. Statistical analyses suggest that for crop pests, resistance evolves most readily in those with an intermediate number of generations (four to ten) per year that feed either by chewing or by sucking on plant cell contents.

Resistant pest species outnumber resistant beneficial species such as predators and parasitoids by more than twenty to one. This pattern probably reflects limited attention devoted to resistance in beneficials as well as biological differences between beneficials and pests. Available evidence contradicts the hypothesis that natural enemies evolve resistance less readily because intrinsic levels of detoxification enzymes are lower in predators and parasitoids than in pests. An alternative hypothesis with more support is that natural enemies evolve resistance less readily because they suffer from food limitation following insecticide sprays that severely reduce abundance of their prey or hosts.
According to Georghiou (1986), pesticide resistance occurs in at least 100 species of plant pathogens, 55 species of weeds, 5 species of rodents, and 2 species of nematodes. This article focuses on resistance to insecticides in more than 500 species of insects and mites.
Sukhoruchenko and Dolzhenko (2008), presents the results of long-term monitoring of insecticide resistance in populations of agricultural pests in Russia. Over the last 45 years, resistance developments were recorded for 36 arthropod pest species in 11 agricultural crops and pastures in relation to nearly all commonly used plant protection products. Development of group, cross and multiple resistance has been revealed in populations of many economically important pests. Toxicological and phenotypical (for Colorado potato beetle) methods have been devised to monitor the development of pesticide resistance. Based on experience over the last century, systems aimed at preventing the development of pest resistance to insecticides and acaricides are elaborated. These systems are based on resistance monitoring and using plant protection measures which minimize the toxic pressure on agroecosystems.

3. Mechanisms of insecticide resistance in insects

There are several ways insects can become resistant to crop protection products, and pests often exhibit more than one of these mechanisms at the same time.

- **Behavioral resistance:** Resistant insects may detect or recognize a danger and avoid the toxin. This mechanism of resistance has been reported for several classes of insecticides, including organochlorines, organophosphates, carbamates and pyrethroids. Insects may simply stop feeding if they come across certain insecticides, or leave the area where spraying occurred (for instance, they may move to the underside of a sprayed leaf, move deeper in the crop canopy or fly away from the target area) (www.irac-online)
- **Penetration resistance:** Resistant insects may absorb the toxin more slowly than susceptible insects. Penetration resistance occurs when the insect's outer cuticle develops barriers which can slow absorption of the chemicals into their bodies. This can protect insects from a wide range of insecticides. Penetration resistance is frequently present along with other forms of resistance, and reduced penetration intensifies the effects of those other mechanisms.
- **Metabolic resistance:** Resistant insects may detoxify or destroy the toxin faster than susceptible insects, or quickly rid their bodies of the toxic molecules. Metabolic resistance is the most common mechanism and often presents the greatest challenge. Insects use their internal enzyme systems to break down insecticides. Resistant strains may possess higher levels or more efficient forms of these enzymes. In addition to being more efficient, these enzyme systems also may have a broad spectrum of activity (i.e., they can degrade many different insecticides).

- **Altered target-site resistance:** The site where the toxin usually binds in the insect becomes modified to reduce the insecticide's effects. This is the second most common mechanism of resistance.

There are four major mechanisms of resistance in insects. These are:

1. Increased metabolism to non-toxic products
2. Decreased target site sensitivity
3. Decreased rates of insecticide penetration
4. Increased rates of insecticide excretion

Of these four categories the first two are by far the most important.

Metabolic resistance: The normal enzymatic metabolism of insect is modified to increase insecticide detoxification or prevent activation of insecticides.

The enzymes responsible for detoxification of xenobiotics in living organisms are transcribed by members of large multigene families of esterases, oxidases, and GST. Glutathione transferases (GSTs) are a diverse family of enzymes found ubiquitously in aerobic organisms. They play a central role in the detoxification of both endogenous and xenobiotic compounds and are also involved in intracellular transport, biosynthesis of hormones and protection against oxidative stress. Interest in insect GSTs has primarily focused on their role in insecticide resistance. GSTs can metabolize insecticides by facilitating their reductive dehydrochlorination or by conjugation reactions with reduced glutathione, to produce water-soluble metabolites that are more readily excreted. In addition, they contribute to the removal of toxic oxygen free radical species produced through the action of pesticides. Annotation of the *Anopheles gambiae* and *Drosophila melanogaster* genomes has revealed the full extent of this enzyme family in insects (Enayati et al,2005). Perhaps the most common resistance mechanisms in insects are modified levels or activities of esterase detoxification enzymes that metabolize (hydrolyze ester linkages) a wide range of insecticides. These esterases comprise six families of proteins belonging to the α/ß hydrolase fold superfamily. In Diptera, they occur as a gene cluster on the same chromosome. Individual members of the gene cluster may be modified in instances of insecticide resistance, for example, by changing a single amino acid that converts the specificity of an esterase to an insecticide hydrolase or by existing as multiple-gene copies that are amplified in resistant insects (the best studied examples are the B1 and A2-B2 amplicons in *Culex pipiens* and *C. quinquefasciatus* (Brogdon and McAllister,1998).

The cytochrome P450 oxidases (also termed oxygenases) metabolize insecticides through O-, S-, and N-alkyl hydroxylation, aliphatic hydroxylation and epoxidation, aromatic hydroxylation, ester oxidation, and nitrogen and thioether oxidation. The cytochrome P450s belong to a vast superfamily. Of the 62 families of P450s recognized in animals and plants, at least four (families 4,6,9,18) have been isolated from insects. The insect P450 oxidases responsible for resistance have belonged to family 6, which, like the esterases, occur in Diptera as a cluster of genes. Members of the cluster may be expressed as multiple (up to five) alleles. Enhanced levels of oxidases in resistant insects result from constitutive overexpression rather than amplification. The mechanisms of oxidase overproduction in resistance are under extensive investigation and appear to result from both cis- and trans-acting factors, perhaps associated with the phenomenon of induction ((Brogdon and McAllister,1998).

Altered target site: The site of action has been altered to decrease sensitivity to toxic attack. Alterations of amino acids responsible for insecticide binding at its site of action cause the insecticide to be less effective or even ineffective. The target of organophosphorus (OPs) (e.g., malathion, fenitrothion) and carbamate (e.g., propoxur, sevin) insecticides is

acetylcholinesterase in nerve synapses, and the target of organochlorines (DDT) and synthetic pyrethroids are the sodium channels of the nerve sheath. DDT-pyrethroid cross-resistance may be produced by single amino acid changes (one or both of two known sites) in the axonal sodium channel insecticide-binding site. This cross-resistance appears to produce a shift in the sodium current activation curve and cause low sensitivity to pyrethroids. Similarly, cyclodiene (dieldrin) resistance is conferred by single nucleotide changes within the same codon of a gene for a γ-aminobutyric acid (GABA) receptor. At least five point mutations in the acetylcholinesterase insecticide-binding site have been identified that singly or in concert cause varying degrees of reduced sensitivity to OPs and carbamate insecticides.

Physical resistance mechanisms: The pickup or intake of toxic agent is slowed or reduced by modification to the insect skeleton, or the rate of excretion of the toxic compound is increased.

4. Insecticide resistance detection techniques

The mode of action of the insecticides, duration life cycle, clutch size and availability of host determine rate of evolution of resistance. Documenting the dynamics of resistance plays another important role in the approach of its mitigation. Reliable, quick and effective techniques to distinguish between susceptible and resistant individuals are necessary (Gunning,1993 and Brown,1981).

There are several phenogenetic methods available to diagnose resistance in populations of pest species which enable the assessment of how shifts in composition and structure of a population caused by pesticides, may affect its development geographically and over time. Among these, easy-to-use toxicological methods have gained the most recognition worldwide. They enable the determination of levels of population susceptibility to pesticides used, in relation to the ratio of resistant and susceptible genotypes. In 2004 under the aegis of the Commission on resistance, a method manual was published: 'Monitoring the resistance to pesticides in populations of arthropod pests'. Methods included in this manual enable scientists to evaluate development of resistance in populations of 37 species of insects and mites of great practical importance for agricultural practice and medicine. At present, researchers are trying to identify easy-to-see visual morphological characters which could be used for the diagnosis of resistance. In order to achieve this, adults from populations under investigation are sampled and fractions of different morphotypes (morphs) are determined. Each morphotype recognized is then tested from the viewpoint of its susceptibility to toxicants used (Benkovskaya et al., 2000; Vasilyeva et al., 2004, 2005; Fasulati, 2005). The frequency of occurrence of different morphs in the Colorado potato beetle has been shown to be related to their susceptibility to pyrethroids. This has enabled a rapid method to be devised for revealing the resistance to pyrthroids in populations of the pest immediately after appearance of overwintered adults in potato crops (Sukhoruchenko et al., 2006). The above method allows potato growers to rationally schedule the use of these pesticides in seasonal application charts.

5. Insecticide resistance detection methods

The primary mechanisms of resistance are decreased target site sensitivity and increased detoxification through metabolism or sequestration. Target sites are the molecules in insects

that are attacked by insecticides. Decreased target site sensitivity is caused by changes in target sites that reduce binding of insecticides, or that lessen the damage done should binding occur. Metabolism involves enzymes that rapidly bind and convert insecticides to nontoxic compounds. Sequestration is rapid binding by enzymes or other substances with very slow or no processing. Reduced insecticide penetration through the cuticle, and behavioral changes that reduce exposure to insecticide are also mechanisms of resistance. Different mechanisms can occur within an individual insect, sometimes interacting to provide extremely high levels of resistance.
Resistance can be determined by using conventional standard bioassay methods published by International Resistance Action Committee (IRAC) and biochemical, immunological and molecular methods.

1) Conventional Detection Methods

The standard method of detection in to take sample of insects from the field and rear them through to the next generations. Larvae or adults are tested for resistance by assessing their mortality after exposure to a range of doses of an insecticide. For susceptible and field populations, LD_{50} or LC_{50} values were calculated by using probit analysis
The results are compared with those from standard susceptible populations. These method includes some differences for the different pest species. These methods are published by Insecticide Resistance Action Committee (IRAC).
The other traditional method of detecting insecticide resistance is to expose individual insects to a diagnostic single dose for a set time period in a chamber impregnated with the insecticide or on a filter paper impregnated with the insecticide. These tests only give an indication of the presence and frequency of resistance and limited information can be gained as to the resistance mechanism.
Evolution of resistance is most often based on one or a few genes with major effect. Before a susceptible population is exposed to an insecticide, resistance genes are usually rare because they typically reduce fitness in the absence of the insecticide. When an insecticide is used repeatedly, strong selection for resistance overcomes the normally relatively minor fitness costs associated with resistance when the population is not exposed to insecticide.

2) Biochemical detection of insecticide resistance

Biocahemical assays/techniques may be used to establish the mechanism involved in resistance. When a population is well characterised some of the biochemical assays can be used to measure changes in resistance gene frequencies in field populations under different selection pressure.

3) Immunological Detection Methods:

This method is available only for specific elevated esterases in collaboration with laboratories that have access to the antiserum. There are no monoclonal antibodies, as yet , available for this purpose.
An antiserum has been prepared against E4 carboxylesterase in the aphid *Myzus persicae* . An affinity purified 1gG fraction from this antiserum has been used in a simple immunoassay to discriminate between the three common resistant variants of *M. persicae* found in the UK field populations (Devonshire et al, 1996).

4) Detection of monooxygenase (cytocrome P450) based insecticide resistance.

The levels of oxidase activity in individual pests are relatively low and no reliable micrtitre plate or dot-blot assay has been developed to measure p450 activity in single insects. The

p450s are also a complex family of enzymes, and it appears that different cytocromes p450s produce resistance to different insecticides.

6. Management of insecticide resistance

Resistance monitoring programme should no longer rely on testing the response to one insecticide, with the intention of switching to another chemical when resistance levels rise above the threshold which affects disease control. Effective resistance management depends on early detection of the problem and rapid assimilation of information on the resistant insect population so that rational pesticide choices can be made.

After a pest species develops resistance to a particular pesticide, how do you control it? One method is to use a different pesticide, especially one in a different chemical class or family of pesticides that has a different mode of action against the pest. Of course, the ability to use other pesticides in order to avoid or delay the development of resistance in pest populations hinges on the availability of an adequate supply of pesticides with differing modes of action. This method is perhaps not the best solution, but it allows a pest to be controlled until other management strategies can be developed and brought to bear against the pest. These strategies often include the use of pesticides, but used less often and sometimes at reduced application rates.

The goal of resistance management is to delay evolution of resistance in pests. The best way to achieve this is to minimize insecticide use. Thus, resistance management is a component of integrated pest management, which combines chemical and non chemical controls to seek safe, economical, and sustainable suppression of pest populations. Alternatives to insecticides include biological control by predators, parasitoids, and pathogens. Also valuable are cultural controls (crop rotation, manipulation of planting dates to limit exposure to pests, and use of cultivars that tolerate pest damage) and mechanical controls (exclusion by barriers and trapping).

Because large-scale resistance experiments are expensive, time consuming, and might worsen resistance problems, modeling has played a prominent role in devising tactics for resistance management. Although models have identified various strategies with the potential to delay resistance, practical successes in resistance management have relied primarily on reducing the number of insecticide treatments and diversifying the types of insecticide used. For example, programs in Australia, Israel, and the United States have limited the number of times and periods during which any particular insecticide is used against cotton pests.

Resistance management requires more effective techniques for detecting resistance in its early stages of development.

Pest resistance to a pesticide can be managed by reducing selection pressure by this pesticide on the pest population. In other words, the situation when all the pests except the most resistant ones are killed by a given chemical should be avoided. This can be achieved by avoiding unnecessary pesticide applications, using non-chemical control techniques, and leaving untreated refuges where susceptible pests can survive.[17][18] Adopting the integrated pest management (IPM) approach usually helps with resistance management.

When pesticides are the sole or predominant method of pest control, resistance is commonly managed through pesticide rotation. This involves alternating among pesticide classes with different modes of action to delay the onset of or mitigate existing pest resistance.[19]

Different pesticide classes may have different effects on a pest. The U.S. Environmental Protection Agency (EPA or USEPA) designates different classes of fungicides, herbicides and insecticides. Pesticide manufacturers may, on product labeling, require that no more than a specified number of consecutive applications of a pesticide class be made before alternating to a different pesticide class.

Tank mixing pesticides is the combination of two or more pesticides with different modes of action in order to improve individual pesticide application results and delay the onset of or mitigate existing pest resistance.

7. References

Anonymous, (2007). Grapes.msu.edu. How pesticide resistance develops. Except from: Larry Gut,Annemiek Schilder, Rufus Isaacs and Patricia McManus. *Fruit Crop Ecology and Management*, Chapter 2: "Managing the Community of Pests and Beneficials." Retrieved on September 15, 2007.

Anonymous, (2011). http://science.jrank.org/pages/48691/Pesticide-Resistance.html".

Benkovskaya GV, Novitskaya OP, Leontyeva TL & Nikolenko AG (2000). [Volaton selected phenes of Colorado beetle].
Современноесостояниепроблемырезистентностивредитедей, возбудителейболезнейисорняковкпестицидамв Россииисопредельныхстранахнарубеже XXI века. *Мат. девятогосовещания (СПб.)*, 84 (in Russian).

Berlinger,R.G. (1996). Pest Resistance to Pesticides. Department of Entomology, Clemson University. printed March 1996.

Brogdon, W.G. and McAllister,J.C. (1998). Insecticide Resistance and Vector Control. Emerging Infecticious Disease, 4(4), October-Dicember,1998.

Brown, T.M. (1981).Counter measures for insecticideresistance. Bull.Entomol. Soc. America., 27:198-202.

Devonshire,A.L., Moores, G.D. and ffrench-Constant, R.H. (1986). Detection os insecticide resistance by immunological estimation of carboxylesterase activity in *Myzus persicae* (Sulzer) and cross reaction of antiserum with *Phorodon numuli* (Schrank) (Hemiptera: Aphidae) . Bull.Ent.Res. 76,97-107.

Enayati,A.A., Ranson,H. and Hemingway,J. (2005).Insect glutathione transferases and insecticide resistance. *Insect Molecular Biology, 14(1):3-8.*

Fasulati, S.R. (2005). Intraspecific variability and a role of anthropogenic factors of microevolution of a sunn pest. Мат. Второгосъездапозащитерастений 'Фитосанитарноеоздоровлениеэкосистем',симпозиум 'Резистентностьвредныхорганизмовкпестицидам' (СПб.), 74–77 (in Russian).

Georghiou, G. P. (1986), The magnititude of resistance problem. In pesticide Resistance: Stratecies and Tactics for management, pp14-43. Washington, D.C., 1986.

Gunning, R.V. (1993). Comparison of the two bio-assay techniques for larvae of helicoverpa sp. (Lepidoptera:Noctuidae). J. Econ. Entomol., 86:234-238.

PBS (2001), Pesticide resistance. Retrieved on September 15, 2007.

Sukhoruchenko, G. I. and Dolzhenko, V. I. (2008). Problems of resistance development in arthropod pests of agricultural crops in Russia. EPPO Bulletin, 38 (1): 119-126. Published online 12 Mar 2008.

Vasilyeva TI, Ivanov GP, Sukhoruchenko GI, Ivanov SG & Shevchenko NM (2004) [Phenotypic variation in the Colorado potato beetle populations under influence of pyrethroids and other factors]. ХИмИческИЙмеТоДзаЩИТырасТенИЙ(СПб.), 43–45 (in Russian).

Vasilyeva TI, Ivanov GP, Ivanov SG & Zverev AA (2005) [Changes of phenotypic structure of Colorado beetle populations due to intensive insecticide use]. *Мат. Второгосъездапозащитерастений'Фитосанитарноеоздоровлениеэкосистем', симпозиум 'Резистентностьвредныхорганизмовкпестицидам' (СПб.)*, 14–15 (in Russian).

Part 4

Analytical Methods Used

22

Review on Current Analytical Methods with Chromatographic and Nonchromatographic Techniques for New Generation Insecticide Neonicotinoids

Eiki Watanabe
National Institute for Agro-Environmental Sciences
Tsukuba,
Japan

1. Introduction

Neonicotinoid insecticides are a major group and the newest group among insecticides. They permeate the entire body of a plant and show excellent effects for the control of diseases and harmful insects. The history of neonicotinoid insecticides from development to their market release dates back to the late 1800s. Nicotine (Fig. 1), an alkaloid in tobacco leaves, is an early neonicotinoid insecticide that has been used as a natural insecticide, but it is extremely toxic to mammals (LD_{50} = 50–60 mg/kg; Tomizawa & Casida, 2005). Therefore, studies using nicotine as a model compound have been conducted actively to develop new pesticides with highly selective toxicity. In the 1970s, Shell developed nithiazine (Fig. 1), which showed strong insecticide activity (Soloway et al., 1978, 1979), although the compound was unstable in its application and remained commercially unavailable. Subsequently, the development of nithiazine derivatives was continued based on the relation between the chemical structure of nicotine compounds and insecticide activity. Eventually, Nihon Tokushu Noyaku Seizo (currently Bayer Crop Science) developed imidacloprid (Fig. 1) (Shiokawa et al., 1994). Subsequent to imidacloprid, acetamiprid (Nippon Soda Co. Ltd.), nitenpyram (Takeda Chemical Industries, currently Sumitomo Chemical Takeda Agro Co.), thiamethoxam (Ciba, currently Syngenta), thiacloprid (Bayer Crop Science), dinotefuran (Mitsui Chemicals Inc.) and clothianidin (Takeda Chemical Industries, currently Sumitomo Chemical Takeda Agro Co.) have been released on the market (Tomlin, 2003) with the subsequent new and recent development of imidaclothiz in China (Nantong Jiangshan Agrochemical and Chemical Co. Ltd.) (Fig. 1).

Neonicotinoid insecticides express insecticide activity by acting on the nicotinic acetylcholine receptor, nAChR, which is present on the postsynaptic membrane of the insect nerve. Excellent insecticide effects are expressed on hemipteran pest species including aphids, whitefly, and planthoppers by this mechanism of action. Although nAChR is present in both insects and mammals, neonicotinoid insecticides that act on them are highly selectively toxic to insects because the recognition site of insect nAChR is lipid-soluble, whereas mammalian nAChR must be ionized to a high degree. It is considered that the

selectivity results from the fact that neonicotinoid insecticides are not, unlike nicotine, fully ionic compounds. Therefore, they are transferred easily in lipophilic insect body fluids to reach nAChR to express the action, although affinity to nAChR is low in mammals (Kagabu, 1996; Shiokawa et al., 1994; Tomizawa, 1994; Tomizawa & Casida, 2003, 2005).

Neonicotinoid insecticides have come into worldwide use because, as described above, they have highly selective toxicity. Their toxicity to mammals, fish, and birds is low. They show a superior effect to control diseases and harmful insects that are resistant to insecticides including organophosphorus insecticides, carbamate insecticides, and synthetic pyrethroid insecticides. Recently however, it has been suggested that neonicotinoid insecticides are a possible cause of colony collapse disorder (CCD), i.e., sudden disappearance of bees that are pollinators of vegetables and fruits in modern agriculture (Decourtye & Devillers, 2010; El Hassani et al., 2008; Girolami et al., 2009; Iwasa et al., 2004; Mommaerts et al., 2010; Nauen et al., 2001). Studies have been undertaken to elucidate the relation between CCD and neonicotinoid insecticides, but the cause of CCD remains unclear.

As described above, neonicotinoid insecticides have come into use as insecticides of the next generation to replace classical insecticides such as organophosphorus insecticides for the stable supply of various crops. However, the influence on the ecosystem related to useful insects such as bees has been noted. Further studies must be undertaken from various viewpoints such as food safety including crops, environment and ecological influence, i.e., for risk management and risk assessment.

In this chapter, the author reports analytical methods that constitute the underlying technology that is indispensable for studies of risk management and risk assessment of neonicotinoid insecticides in the form of review to systemize cases reported to date, as well as to organize the trend, current situation, and future directions observed in the overview of respective analytical methods.

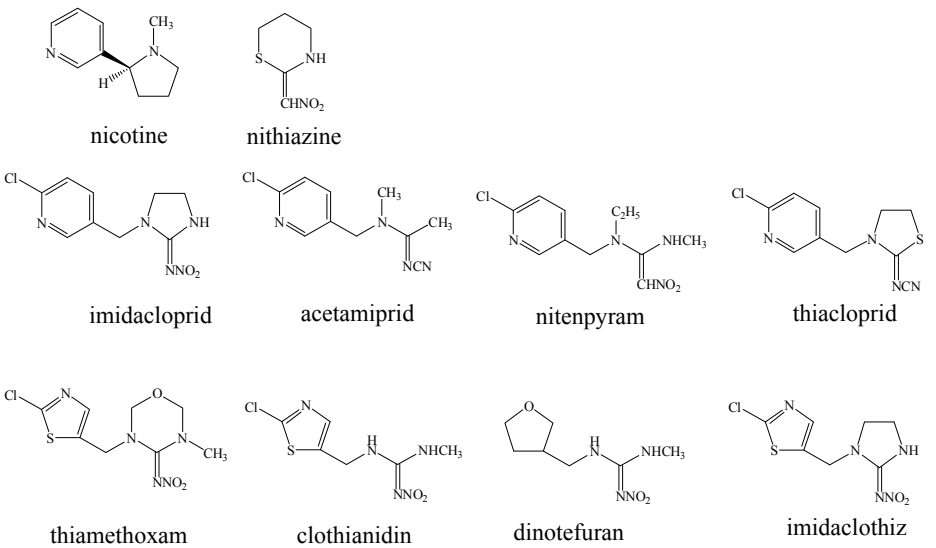


Fig. 1. Chemical structures of nicotine and neonicotinoid insecticides having nitromethylene moiety (C=CHNO$_2$), nitroguanidine moiety (C=NNO$_2$) and cyanoamidine moiety (C=NCN).

2. Analytical methods based on chromatographic techniques

Chromatography is a powerful tool for the determination of pesticides that might remain in widely various matrices such as food including crops, living bodies, and the environment. It also is used as an important method for the analysis of neonicotinoid insecticides, which were introduced into the environment about twenty years ago as the successor of organophosphorus, carbamate, and synthetic pyrethroid insecticides. This section refers to sample pre-treatments including extraction and clean-up needed before chromatographic determination and gives an exhaustive summary of the trend related to the development of residue analysis of neonicotinoid insecticides by chromatography.

2.1 Sample pre-treatment procedures prior to chromatographic determination

To analyze minute amounts of residual pesticides in complex matrices accurately, sample pre-treatment procedures must be conducted before chromatographic determination. It is no exaggeration to say that the results of the procedures have a decisive influence on the reliability of the data measured. This also applies to the analysis of neonicotinoid insecticides. In pesticide residue analyses conducted in the mid-1990s, when imidacloprid became available on the market, gas chromatograph (GC) equipped with so-called element-selective detectors, for example, electron capture ionization detector (ECD) for detection of pesticides with halogen atoms such as organochlorine insecticides and synthetic pyrethroid insecticides or flame photometric detector (FPD) for detection of pesticides with phosphorus atoms or sulfur atoms such as organophosphorus insecticides was mainly used. On the other hand, high-performance liquid chromatograph (HPLC) equipped with UV detectors or diode array detectors (DAD) were used for detection of pesticides that were unstable to heat. It is important in all measurement methods to conduct sample pre-treatment procedures to obtain accurate measurement data.

Table 1 shows that sample pre-treatment procedures roughly consist of (1) extraction of the target pesticide from the sample and (2) separation of the target pesticide from the extract and clean-up.

For (1) extraction of neonicotinoid insecticides, shaking extraction with organic solvents such as acetone, acetonitrile, or methanol (Baskaran et al., 1997; de Erenchun et al., 1997; Mohan et al., 2010; Tokieda et al., 1997b, 1998; Watanabe et al., 2007), blending extraction with a homogenizer (Agüera et al., 2004; Blasco et al., 2002a, 2002b; Di Muccio et al., 2006; Fernandez-Alba et al., 1996, 2000; Ferrer et al., 2005; Hengel & Miller, 2008; Hernández et al., 2006; Ishii et al., 1994; Jansson et al., 2004; Kamel et al., 2010; Mateu-Sánchez et al., 2003; Obana et al., 2002, 2003; Sannino et al., 2004; Ting et al., 2004; Tokieda et al., 1997a, 1997b; Venkateswarlu et al., 2007), and ultrasonic extraction (Bourgin et al., 2009; García et al., 2007; Ishii et al., 1994; Liu et al., 2005, 2010; Mayer-Helm, 2009; Rancan et al., 2006a, 2006b; Zhang et al., 2010) are commonly used. In addition to these, Bourgin et al. (2009) extracted five insecticides including imidacloprid from seeds coated with acetonitrile and Xiao et al. (2011) extracted seven neonicotinoid insecticides from bovine tissues with water by accelerated solvent extraction (ASE) to give quantitative extraction efficiency. For extraction from liquid samples such as water, milk and wine, solid-phase extraction (SPE) packed with e.g. C_{18} or diatomaceous earth (Baskaran et al., 1997; Economou et al., 2009; Ferrer & Thurman, 2007; Pirard et al., 2007; Seccia et al., 2005, 2008; Zhou et al., 2006) and liquid–liquid extraction (Galera et al., 1998; Vilchez et al., 1996, 2001) are used. Moreover, matrix solid-phase dispersion (MSPD) was first reported in 1989. A small amount of solid or semisolid sample

was blended with adsorbents such as C_{18} to be packed in a disposable plastic column. It was then extracted with a suitable organic solvent (Barker et al., 1989). The method has been applied to the extraction of pesticides, pharmaceuticals, and antibiotics since then (Barker, 2000a, 2000b). MSPD is also applied to the extraction of neonicotinoid insecticides from crop, honey, and fruit juice samples (Blasco et al., 2002a; Pous et al., 2001; Radišić et al., 2009; Totti et al., 2006).

In any event, the first obstacle in the development of analytical methods for pesticide residues is to secure extraction efficiency from measurement samples. It is probably important to select extraction conditions that are suitable for the physicochemical features of the target pesticide and for the characteristics of the measurement sample.

In the process of (2) clean-up, classical liquid–liquid partitioning and column chromatography have been used. However, because of concern over the health impact of the use of organic solvents in large quantities on analytical staff as well as the environmental load, the strong need exists for the reduction of organic solvents used in sample pre-treatment procedures (Wan & Wong, 1996). SPE packed with widely various adsorbents such as silica gel, Florisil, C_{18}, polymeric materials, graphitized carbon black (GCB), and ion exchange resin has come into common use these days, making a great contribution to reduction of the use of organic solvents (Fritz & Macka, 2000).

In the 2000s, marked technical innovation in analytical instruments has taken place: LC-MS and LC-MS/MS have been brought into use for the determination of neonicotinoid insecticides. These methods have higher measurement sensitivity than classical HPLC-UV and HPLC-DAD. Moreover, in many cases, sample pre-treatment procedures have reportedly included only the extraction and dilution of the extract, with subsequent measurement, without even the need for additional clean-up (Table 1). The most common extraction procedure is the following: a measurement sample is homogenized; then it is dehydrated and extracted with ethyl acetate and anhydrous sodium sulfate with subsequent solvent evaporation and then LC-MS determination (Blasco et al., 2002a, 2002b; Fernández-Alba et al., 2000) or LC-MS/MS determination (Agüera et al., 2004; Jansson et al., 2004; Venkateswarlu et al., 2007).

In 2003, Anastassiades et al. (2003) reported fast, simple and easy sample pre-treatment procedures by extraction with acetonitrile and dispersive SPE. The method that was presented is known as a quick, easy, cheap, effective, robust, and safe (QuEChERS) method, which is a breakthrough in which the sample pre-treatment is completed via acetonitrile extraction, dehydration, and salting-out with anhydrous magnesium sulfate and sodium chloride. Thereafter, dehydration and clean-up of the extract are done using dispersive SPE with anhydrous magnesium sulfate and primary secondary amine (PSA), which is a weak anion exchange adsorbent. Reportedly dispersive SPE using PSA is extremely effective for the removal of organic acids, polar dye components, and saccharides (Anastassiades et al., 2003). QuEChERS is very different from general sample pre-treatment procedures by organic solvent extraction, re-extraction with the organic solvent phase, and clean-up with SPE in that no process of concentration (evaporation of solvents) is needed. The concentration process, a procedure that must be conducted after processes such as extraction and clean-up, unexpectedly accounts for a large percentage of the sample pre-treatment time. Therefore, QuEChERS is a technology that contributes much to speeding up and simplification of sample pre-treatment procedures. In addition, the amounts of organic solvents used are extremely as small: about 10 mL of acetonitrile per sample. For those reasons, it can be concluded that the technology has met all of the requirements described above (Wan & Wong, 1996).

GC						
Analyte(s)	Sample(s)	Extraction	Clean-up	Additional procedure prior to GC analysis	Determination	Ref.
Imidacloprid	Water	Mechanical shaking with chloroform	None	Hydrolysis in basic medium by application of heat	GC–MS	Vilchez et al., 1996
	Soil	Ultrasonic extraction with water and mechanical shaking with chloroform				
Imidacloprid	Tomato, cucumber, pepper and green bean	Ultrasonic extraction with water and mechanical shaking with chloroform	None	Hydrolysis in basic medium by application of heat	GC–MS	Navalón et al., 1997
Acetamiprid and 4 metabolites	Cabbage, green pepper, eggplant, potato, apple, orange, grape, strawberry, cucumber and radish (root, leaf)	Homogenization with methanol	Liquid–liquid partition and silica gel column chromatography	Hydrolysis in basic medium by application of heat, oxidation with $KMnO_4$ by application of heat and esterification with CH_2N_2	GC-ECD	Tokieda et al., 1997a
Acetamiprid	Cabbage, potato, radish (leaf, root), grape, orange, apple, strawberry, green pepper and eggplant	Homogenization and mechanical shaking with methanol	Liquid–liquid partition, Florisil column chromatography and C_{18} SPE (optional)	None	GC-ECD	Tokieda et al., 1997b
	Green tea (powder)	Mechanical shaking with methanol				
	Green tea (leachate)	Liquid–liquid extraction with methanol after soak in Boiling water				
3 pesticides including imidacloprid	White pine	Maceration with methanol/0.04% H_2SO_4 (70:30, v/v)	C_{18} SPE and self-prepared Florisil minicolumn	Heptafluorobutyryl derivative	GC–MS	MacDonald & Meyer, 1998
Acetamiprid	Vegetables	Homogenization with ethyl acetate	None	None	GC–MS/MS	Mateu-Sánchez et

					al., 2003
HPLC equipped with detectors except for MS or tandem MS					
Analyte(s)	Sample(s)	Extraction	Clean-up	Determination	Ref.
Imidacloprid	Japanese pear, apple, peach (pulp, peel), grape, radish (root, leaf), cucumber, eggplant, rice grain, rice green, rice straw and potato	Homogenization with acetonitrile/water (80:20, v/v)	Liquid–liquid partition and silica gel column chromatography	HPLC-UV	Ishii et al., 1994
	Soil	Ultrasonic extraction with acetonitrile/water (80:20, v/v)			
Imidacloprid	Pepper, tomato and cucumber	Homogenization with acetone	Liquid–liquid partition and C_{18} SPE	HPLC-DAD	Fernandez-Alba et al., 1996
Imidacloprid	Water	Extraction with methanol from C_{18} SPE	None	HPLC-UV	Baskaran et al., 1997
	Soil	Mechanical shaking with acetonitrile/water (80:20, v/v)	None		
Imidacloprid and 6-chloronicotinic acid	Soil	Mechanical shaking with acetonitrile/methanol/water (3:2:2, v/v)	None	HPLC-pulsed amperometric detector	de Erenchun et al., 1997
Imidacloprid and 6-chloronicotinic acid	Groundwater	Liquid–liquid extraction with dichloromethane	None	HPLC-DAD	Galera et al., 1998
Acetamiprid and 2 metabolites	Soil	Mechanical shaking with methanol/0.1 M NH_4Cl (8:2, v/v)	Liquid–liquid partition and Extrelut SPE packed with diatomaceous earth material	HPLC-UV	Tokieda et al., 1998
6-chloronicotinic acid		Mechanical shaking with methanol/0.1 M NH_4Cl (8:2, v/v) and methanol/0.5 M NaOH (8:2, v/v)	C_{18} SPE and liquid–liquid partition		
Imidacloprid and 6-chloronicotinic acid	Greenhouse air	Trap with Amberlite XAD-2 and desorption with acetonitrile and phosphate buffer	None	HPLC-DAD	Frenich et al., 2000
Acetamiprid, imidacloprid and nitenpyram	Cucumber, potato, tomato, eggplant, Japanese radish and grape	Homogenization with acetonitrile	PSA SPE and silica gel SPE	HPLC-DAD	Obana et al., 2002
3 pesticides including imidacloprid and thiamethoxam	Cabbage, tomato, chili, pepper and potato	Microwave-assisted extraction with acetone	Liquid–liquid partition	HPLC-UV	Singh et al., 2004

Imidacloprid	Grape, cantaloupe, strawberry, cucumber, tomatillo, lettuce and bell pepper	Homogenization with ethyl acetate and anhydrous Na_2SO_4	NH_2 and Florisil SPE	HPLC-nitrogen chemiluminescent detector and DAD	Ting et al., 2004
Imidacloprid	Tobacco leaf	Ultrasonic extraction with ethyl acetate	Liquid–liquid partition	HPLC-UV	Liu et al., 2005
Thiamethoxam	Honeybee	Ultrasonic extraction with acetone	None	HPLC-ECD	Rancan et al., 2006a
Imidacloprid and 2 metabolites	Bee and filter paper	Ultrasonic extraction with acetone	None	HPLC-ECD	Rancan et al., 2006b
	Maize leaf		Hydromatrix SPE packed with diatomaceous earth material		
Acetamiprid, imidacloprid and thiamethoxam	Water	Extraction with methanol from SPE packed with multiwalled carbon nanotubes	None	HPLC-UV	Zhou et al., 2006
Imidacloprid and 6-chloronicotinic acid	Honeybee	Ultrasonic extraction with acetone	Liquid–liquid partition	HPLC-FLD	García et al., 2007
Acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid and thiamethoxam	Apple, carrot, cucumber, grape, peach, sweet pepper, spinach and tomato	Mechanical shaking with acetone	Chem Elut SPE packed with diatomaceous earth material and GCB/NH_2 SPE	HPLC-DAD	Watanabe et al., 2007
Acetamiprid, imidacloprid, thiacloprid and thiamethoxam	Bovine milk	Extraction with dichloromethane from Chem Elut SPE packed with diatomaceous earth material	None	HPLC-DAD	Seccia et al., 2008
5 pesticides including imidacloprid	Coated seeds of wheat and corn	Ultrasonic extraction with acetonitrile	None	HPLC-UV	Bourgin et al., 2009
		ASE with acetonitrile			
Imidacloprid	Orange juice, apple juice and mixture of pineapple and pear juice	None	Dilution of 0.05 M sodium dodecyl sulfate	HPLC-DAD	Chin-Chen et al., 2009
Acetamiprid, imidacloprid and thiacloprid	Cotton seed	Mechanical shaking with acetone/water (80:20, v/v)	C_{18} SPE	HPLC-UV	Mohan et al., 2010
HPLC equipped with MS or tandem MS					
Analyte(s)	Sample(s)	Extraction	Clean-up	Determination	Ref.
5 pesticides including imidacloprid	Pear and tomato	Homogenization with ethyl acetate and anhydrous Na_2SO_4	None	LC-MS	Fernández-Alba et al., 2000
5 pesticides including imidacloprid	Strawberry, orange, potato, pear and melon	Extraction with dichloromethane from MSPD with C_8	None	LC-MS	Pous et al., 2001
10 pesticides	Orange	Extraction with	None	LC-MS	Blasco et al.,

including imidacloprid		dichloromethane from MSPD with C_8 Stir bar sorptive extraction after homogenization with methanol and water Homogenization with ethyl acetate and anhydrous Na_2SO_4			2002a
4 pesticides including imidacloprid	Peach and nectarine	Homogenization with ethyl acetate and anhydrous Na_2SO_4	None	LC-MS	Blasco et al., 2002b
Acetamiprid, imidacloprid, nitenpyram, thiacloprid and thiamethoxam	Bell pepper, cucumber, eggplant, grape, grapefruit, Japanese radish (root, leaf), peach, pear, potato, rice and tomato	Homogenization with methanol	GCB SPE	LC-MS	Obana et al., 2003
Imidacloprid	Soil	Mixing with methanol/0.05% NH_4OH (3:1, v/v)	None	LC-MS/MS	Bonmatin et al., 2003
	Maize, rape, wheat and sunflower	Grinding with methanol/0.05% H_2SO_4 (4:1, v/v)	C_{18} SPE		
	Pollen	Mixing with ethanol/water (75:25, v/v)	None		
57 pesticides including imidacloprid and acetamiprid	Fruits and vegetables	Homogenization with ethyl acetate and anhydrous Na_2SO_4	None	LC-MS/MS	Jansson et al., 2004
24 pesticides including imidacloprid	Apple puree, concentrated lemon juice and tomato puree	Homogenization with acetone and ethyl acetate/cyclohexane (50:50, v/v)	None	LC-MS/MS	Sannino et al., 2004
17 pesticides including acetamiprid, imidacloprid and thiacloprid	Pepper, lettuce and eggplant	Homogenization with ethyl acetate and anhydrous Na_2SO_4 under alkaline	None	LC-MS/MS	Agüera et al., 2004
Acetamiprid, imidacloprid, thiacloprid and thiamethoxam	Honey	Extraction with dichloromethane from Extrelut SPE packed with diatomaceous earth material	None	LC-MS	Fidente et al., 2005
Acetamiprid, imidacloprid, thiacloprid and thiamethoxam	Drinking water	Extraction with ethyl acetate/methanol (50:50, v/v) from poly(styrene-divinylbenzene) SPE	None	LC-MS	Seccia et al., 2005
Acetamiprid, imidacloprid and thiacloprid	Cucumber, tomato, lettuce and pepper	Homogenization with ethyl acetate	None	LC-TOF-MS	Ferrer et al., 2005
146 pesticides including acetamiprid,	Lettuce and orange	QuEChERS method Shaking by hand with acetonitrile	Dispersive PSA SPE	LC-MS/MS	Lehotay et al., 2005

imidacloprid, thiacloprid and thiamethoxam					
Acetamiprid, imidacloprid, thiacloprid and thiamethoxam	Apricot, celery, courgette, peach and pear	Homogenization with acetone and extraction with dichloromethane from Extrelut SPE packed with diatomaceous earth material	None	LC-MS	Di Muccio et al., 2006
52 pesticides including acetamiprid, imidacloprid and thiacloprid	Lemon, raisin, tomato and avocado	Homogenization with methanol/water (80:20, v/v) containing 0.1% formic acid	HLB (hydrophilic–lipophilic balanced copolymer) SPE	LC-MS/MS	Hernández et al., 2006
6 pesticides and metabolites including imidacloprid	Honeybee	Extraction with dichloromethane/methanol (85:15, v/v) from MSPD with C_{18}	None	LC-MS	Totti et al., 2006
52 pesticides including acetamiprid, imidacloprid and thiacloprid	Potato, orange and cereal-based baby food	Acetate buffered QuEChERS method			
		Shaking with acetonitrile containing 1% acetic acid	Dispersive PSA SPE	UPLC-MS/MS	Leandro et al., 2007
19 pesticides and metabolites including imidacloprid	Honey	Extraction with ethyl acetate from Chem Elut SPE packed with diatomaceous earth material	None	LC-MS/MS	Pirard et al., 2007
101 pesticides and metabolites including acetamiprid, imidacloprid, nitenpyram and thiacloprid	Green pepper, tomato, cucumber and orange	QuEChERS method			
		Shaking with acetonitrile	Dispersive PSA SPE	LC-TOF-MS	Ferrer & Thurman, 2007
	Water	Extraction with ethyl acetate from C_{18} SPE	None		
42 pesticides including acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid and thiamethoxam	Raisin, red grape and red wine	QuEChERS method			
		Shaking by hand with acetonitrile	Dispersive PSA SPE	LC-MS/MS	Payá et al., 2007
	Orange and wheat flour	Shaking by hand with acetonitrile	None		
10 pesticides including imidacloprid and thiamethoxam	Grape	Homogenization with ethyl acetate and anhydrous Na_2SO_4	None	LC-MS/MS	Venkateswarlu et al., 2007
160 pesticides including acetamiprid, dinotefuran, imidacloprid, nitenpyram, thiacloprid and thiamethoxam	Tomato, pear and orange	QuEChERS method			
		Shaking by hand with acetonitrile	Dispersive PSA SPE	LC-MS/MS	Kmellár et al., 2008
11 pesticides	Dried hop	Homogenization with	Polymeric styrene-	LC-MS/MS	Hengel &

including imidacloprid		acetonitrile	divinylbenzene methacrylate SPE and NH_2 SPE		Miller, 2008
53 pesticides including acetamiprid, imidacloprid, nitenpyram, thiacloprid and thiamethoxam	Orange, strawberry and cucumber	Buffered QuEChERS method: Vortex mixing with acetonitrile containing 1% acetic acid	None	UPLC-MS/MS	Frenich et al., 2008
	Olive	Vortex mixing with acetonitrile containing 1% acetic acid	Florisil SPE		
46 pesticides including acetamiprid and imidacloprid	White and red wines	Extraction with methanol from HLB SPE	None	LC-MS/MS	Economou et al., 2009
52 pesticides including acetamiprid, imidacloprid and thiamethoxam	Tobacco	Ultrasonic extraction and shaking with methanol after soak in 1% acetic acid	Chem Elut SPE packed with diatomaceous earth material and Florisil SPE	LC-MS/MS	Mayer-Helm, 2009
12 pesticides including acetamiprid	Apple juice, peach juice, orange juice and raspberry juice	Extraction with dichloromethane from MSPD with diatomaceous earth material	None	LC-MS/MS	Radišić et al., 2009
7 pesticides and metabolites including imidacloprid and thiamethoxam	Honey and pollen	Extraction with methanol from Florisil SPE	None	LC-MS/MS	García-Chao et al., 2010
42 pesticides including acetamiprid, clothianidin, imidacloprid, thiacloprid and thiamethoxam	Tea (made tea, tea infusion and spent leaf)	Vortex mixing and homogenization with water, ethyl acetate/cyclohexane (9:1, v/v) and sodium chloride	Dispersive SPE with PSA, GCB and Florisil	LC-MS/MS	Kanrar et al., 2010
Acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid and thiamethoxam	Rice and tea	Ultrasonic extraction with acetonitrile	HLB SPE	UPLC-MS/MS	Liu et al., 2010
	Apple, cabbage, potato, chicken, pork, milk and egg	Vortex shaking with acetonitrile			
Clothianidin, dinotefuran, imidacloprid, thiamethoxam and 8 metabolites	Bee, bee pollen and bee honey	Modified QuEChERS method: Homogenization with water and acetonitrile containing 2% triethylamine	C_{18} SPE	LC-MS/MS	Kamel, 2010
36 pesticides including acetamiprid and imidacloprid	Tea	Ultrasonic extraction with acetonitrile containing 1% acetic acid	Dispersive SPE with PSA and GCB	UPLC-MS/MS	Zhang et al., 2010
22 pesticides	Milk, orange,	Homogenization with	Salting out	UPLC-MS/MS	Kamel et al.,

and metabolites including acetamiprid, clothianidin, dinotefuran, thiacloprid, thiamethoxam	spinach, apple, plum, watermelon, green bean, zucchini, broccoli, strawberry, grape and tomato	acetonitrile containing 1% triethylamine	(optional), C_{18} SPE and Florisil SPE		2010
93 pesticides and mycotoxins including acetamiprid, imidacloprid, thiacloprid and thiamethoxam	Wheat, cucumber and red wine	Acetate buffered QuEChERS method Vortex mixing with acetonitrile containing 1% acetic acid	None	UPLC-MS/MS	Romero-González et al., 2011
150 pesticides including acetamiprid, imidacloprid, nitenpyram, thiacloprid and thiamethoxam	Tomato, pear and orange	Acetate buffered QuEChERS method Shaking by hand with acetonitrile containing 1% acetic acid	Dispersive PSA SPE	LC-MS/MS	Kmellár et al., 2011
70 pesticides including imidacloprid, thiacloprid and thiamethoxam	Water	None	None	LC-MS/MS	Pareja et al., 2011
Acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid and thiamethoxam	Bovine muscle and liver	ASE with water	HLB SPE	LC-MS/MS	Xiao et al., 2011
Acetamiprid, clothianidin, dinotefuran, imidacloprid, thiacloprid and thiamethoxam	Chestnut, shallot, ginger and tea	Vortex mixing with acetonitrile	Activated carbon SPE and HLB SPE	LC-MS/MS	Xie et al., 2011
Other chromatographic technique					
Analyte(s)	Sample(s)	Extraction	Clean-up	Determination	Ref.
Imidacloprid and 6-chloronicotinic acid	Greenhouse air	Trap with Amberlite XAD-2 and desorption with water	None	Capillary electrophoresis (micellar electrokinetic chromatography)-DAD	Carretero et al., 2003

Table 1. Overview of chromatographic techniques for determination of neonicotinoid insecticides.

With the presentation of QuEChERS as a turning point, many reports have described multiresidue analysis of various pesticides including neonicotinoid insecticides (Ferrer & Thurman, 2007; Frenich et al., 2008; Kamel, 2010; Kmellár et al., 2008, 2011; Leandro et al., 2007; Lehotay et al., 2005; Payá et al., 2007; Romero-González et al., 2011). Additionally,

reagents and adsorbents for QuEChERS are marketed as kits, implying that the method is extremely practical as multiresidue analysis for pesticide residues.

2.2 Determination of neonicotinoid insecticides based on chromatographic methods

Chromatographic determination of neonicotinoid insecticides are classifiable into two groups that use HPLC or GC (Table 1). However, because neonicotinoid insecticides are generally degraded by heat, additional processes such as derivatization are necessary for GC determination, making sample pre-treatment procedures more complicated than HPLC determination. Vilchez et al. (1996) and Navalón et al. (1997) used a hydrolyzed compound of imidacloprid [1–(6-chloro-3-pyridylmethyl)imidazolidin-2-one] formed by heat treatment of a measurement sample under alkaline conditions for GC–MS determination of imidacloprid in soil, water, and crops. MacDonald and Meyer (1998) extracted imidacloprid from white pine with water-containing methanol under acidic conditions with diluted sulfuric acid, cleaned up with C_{18} SPE and a self-prepared Florisil minicolumn, heptafluorobutyryl-derivatized, and then subjected to GC–MS determination.

As presented above, it is presumed that utilization of HPLC rather than GC is more advantageous for the determination of neonicotinoid insecticides from the viewpoints of speedup and simplification of sample pre-treatment procedures. It also is readily apparent that most cases reported to date used HPLC for determination (Table 1). In determination by HPLC, both HPLC-UV and HPLC-DAD contributed greatly to analyses of neonicotinoid insecticides until the 2000s, when MS and MS/MS began to become popularly used. Since the report by Ishii et al. (1994), HPLC-UV and HPLC-DAD have been applied to the determination of neonicotinoid insecticides in various matrices (Table 1). In addition to UV and DAD, methods were developed in which imidacloprid and thiamethoxam were separated using a column and then converted into electrochemically active compounds by ultraviolet irradiation to be detected by an electrochemical detector (ECD) (Rancan et al., 2006a, 2006b), or converted into fluorescent substances to be detected using a fluorescence detector (FLD) (García et al., 2007). ECD and FLD are generally more sensitive than UV or DAD. They are applied to residue analysis in the bodies of bees, where determination at low concentrations must be done.

In fact, HPLC-UV and DAD have less measurement sensitivity and selectivity than either LC-MS or MS/MS, which are the most widely used methods today. Therefore, thorough clean-up is indispensable for the pre-treatment procedures of samples consisting of complicated matrices such as crops. Liquid–liquid partition has been used since the initial phase of the market release of imidacloprid (Fernandez-Alba et al., 1996; García et al., 2007; Ishii et al., 1994; Liu et al., 2005; Singh et al., 2004). However, that technique presents problems: e.g. a large amount of organic solvent is used; and an emulsion is formed at the liquid–liquid interface depending on the extraction sample that is used. For that reason, it has been increasingly replaced by clean-up mainly by SPE (Mohan et al., 2010; Obana et al., 2002; Ting et al., 2004; Watanabe et al., 2007). Watanabe et al. (2007) used re-extraction with diatomaceous earth SPE and clean-up with GCB/NH_2 SPE in the development of simultaneous analysis by HPLC-DAD of seven neonicotinoid insecticides released on the market. However, the recovery of nitenpyram was not satisfactory (not more than 40%). According to their discussion, matrix components in the sample affected nitenpyram in some way, leading to the factor of reduced recovery.

In the 2000s, a dramatic increase occurred in the number of cases reported on the residue pesticide analysis by HPLC equipped with quadrupole MS, ion-trap MS, tandem MS (MS/MS), and time-of-flight MS (TOF-MS). Utilization of MS enabled not only the detection of trace pesticide residues in various matrices with high accuracy but also the elucidation of their respective chemical structures.

Obana et al. (2003) reported a method of extracting five neonicotinoid insecticides from 12 crop samples using methanol, clean-up with GCB SPE, and determination by LC-MS. They suggested that the analytical method that was developed was effective as a regular monitoring method. In determination methods including neonicotinoid insecticides by LC-MS/MS, good recovery is obtained in most reported cases. In one important case, quantification was achieved by direct injection of the filtered water sample in the LC-MS/MS determination of 70 pesticides including imidacloprid, thiacloprid, and thiamethoxam in paddy water samples (Pareja et al., 2011). It is exactly the benefit of LC-MS/MS, with which highly sensitive determination is possible.

Ultra-performance liquid chromatography (UPLC) was developed only a few years ago in which the mobile phase can be flown at a high pressure (about 15,000 psi) using a short column of about 50 mm packed with C_{18} of particle size of not more than 2 μm. Its application to pesticide residue analysis has been examined because the utilization of UPLC enables not only the achievement of highly sensitive determination, but also improved high throughput attributable to reduced measurement time as well as substantially smaller amounts of organic solvents (mobile phase) used than in conventional HPLC (Frenich et al., 2008; Kamel et al., 2010; Leandro et al., 2007; Liu et al., 2010; Romero-González et al., 2011; Zhang et al., 2010). Liu et al. (2010) used UPLC-MS/MS to construct simultaneous analyses of seven neonicotinoid insecticides in crops and livestock products, obtaining good recovery except for the lower recovery of nitenpyram (not more than 70%) in some of samples including potatoes and cabbages. Romero-González et al. (2011) constructed highly sensitive ultra-rapid analysis of more than 90 pesticides including acetamiprid, imidacloprid, thiacloprid, and thiamethoxam as well as mycotoxins by the combination of sample pre-treatment by QuEChERS and UPLC-MS/MS.

As described above, the trend in the development of residue analysis of various pesticides including neonicotinoid insecticides by LC-MS and LC-MS/MS can be summarized via the overview of several cases. LC-MS and LC-MS/MS are suitable for highly sensitive determination of only slightly volatile and heat-unstable pesticides. They enable quantification only with extremely simple and rapid sample pre-treatment compared to those of HPLC-UV, HPLC-DAD, and GC with an element-selective detector, which requires complicated sample pre-treatment procedures. Particularly MS/MS can be characterized as an effective method for the structural analysis of the target pesticide and its confirmation because much chemical information can be acquired by obtaining product ions from the precursor ion. Moreover, little interference occurs by matrix components because ions can be selected at will, thereby enabling highly sensitive determination. Recently, newly marketed insecticides tend to be included in the subjects for HPLC determination. Therefore, increasing need is expected for LC-MS and LC-MS/MS in the future.

Although many advantages of LC-MS and LC-MS/MS are described above, the matrix effect must also be noted, which is a problem in the chromatographic determination of pesticide residues that might be present in various samples.

It has been pointed out that, as in GC-ECD and GC-FPD determination, quantitative determination is not possible because of the matrix effect when the sample clean-up is insufficient. Lee and Wylie (1991) have reported interesting observations by which the susceptibility (to the matrix effect) of several GC detectors is examined for individual crop samples. The matrix effect is a phenomenon that is also observed in LC-MS and LC-MS/MS determination; it was shown earlier that when the target pesticide is eluted together with matrix components in the sample, ion suppression or ion enhancement occurs during the ionization process, engendering error in the determination result (Niessen et al., 2006). The matrix effect can be avoided using a matrix-matched standard method or using isotope dilution with isotopically labeled internal standards (Niessen et al., 2006), in addition to thorough sample clean-up. In the matrix-matched standard method, the matrix effect is evaluated through the comparison of the responses of the standard solution and the target pesticide prepared in the measurement sample solution free of the target pesticide; this method is commonly used as a means to correct the matrix effect (Di Muccio et al., 2006; Economou et al., 2009; Ferrer et al., 2005; Ferrer & Thurman, 2007; Fidente et al., 2005; Frenich et al., 2008; Hernández et al., 2006; Kamel, 2010; Kamel et al., 2010; Kanrar et al., 2010; Kmellár et al., 2008; Leanro et al., 2007; Mayer-Helm, 2009; Payá et al., 2007; Pirard et al., 2007; Radišić et al., 2009; Romero-González et al., 2011; Totti et al., 2006; Venkateswarlu et al., 2007; Xiao et al., 2011; Xie et al., 2011).

Xie et al. (2011) reported observation of ion enhancement in dinotefuran, imidacloprid, and thiacloprid, as well as ion suppression in acetamiprid, clothianidin, and thiamethoxam by the matrix effect, and that acetamiprid and thiamethoxam among these were significantly affected. However, because the recovery was improved by correction using the matrix-matched standard method, they emphasized the effectiveness of the method in the avoidance of the matrix effect.

In any event, the matrix effect should be regarded as a common problem of pesticide residue analysis by HPLC and GC; it goes without saying that it is most important to evaluate the matrix effect that might be derived from the subject sample when the development of a new analytical method is attempted or when an established, existing analytical method is applied.

3. Analytical methods based on nonchromatographic techniques

Section 2 summarized the trends up to now in the development of the determination of neonicotinoid insecticides by chromatography. This section refers to analytical methods based on nonchromatographic techniques. The list in Table 2 shows that major nonchromatographic methods include flow injection analysis (FIA), direct MS analysis, and enzyme-linked immunosorbent assay (ELISA), which is an immunochemical determination using an antigen–antibody reaction with high specificity or selectivity. This section summarizes the trend and the current situation of the development of analytical methods for neonicotinoid insecticides using these.

3.1 FIA or MS analysis for direct determination of neonicotinoid insecticides

FIA is a method by which a predetermined amount of a sample solution is injected into carrier solution that flows continuously in a tube, and the target substance is detected or quantified using a detector, as might be done after a chemical reaction. Instruments used in

FIA are generally inexpensive. Furthermore, the method is known to be capable of rapid, easy, and highly sensitive detection of trace substances (Lara et al., 2010; Llorent-Martínez et al., 2011).

Reports on the determination of neonicotinoid insecticides by FIA have targeted imidacloprid to date. In all such studies, methods are constructed by which a measurement sample is irradiated with ultraviolet light for conversion into a fluorescent substance [1–(6-chloro-3-pyridylmethyl)-2-(hydroxyimino)-3,4-didehydroimidalozolidene] which is detected using a spectrofluorometer (Flores et al., 2007; Vílchez et al., 2001). Alternatively, nitrite is detached from imidacloprid to be reduced into nitric oxide by iodide, which is detected by chemiluminescence detection with ozone (Lagalante & Greenbacker, 2007). All FIA methods show measurement sensitivity that is equal to or better than the detection limit of HPLC or GC. For the determination of liquid samples such as water, direct injection is possible with filtration only (Flores et al., 2007; Lagalante & Greenbacker, 2007). Actually, FIA is regarded as making a great contribution to fast, simple, and easy determination of pesticide residues, especially in liquid samples.

García-Reyes et al. (2009) reported rapid in situ qualitative and quantitative analysis of 16 pesticides including nitenpyram and thiacloprid by desorption electrospray ionization MS (DESI-MS) and MS/MS (DESI-MS/MS). In their determination, crop samples were pre-treated according to QuEChERS and the resulting sample solution was applied on the PTFE surface, while the skin of fruit and vegetable samples were fixed on glass slides, and electrospray was applied directly to the sample to ionize target pesticides in the sample. The measurement sensitivity was extremely high: on the order of μg/kg, and the measurement accuracy was comparable to LC-MS. Although such an analytical method remains under development at present, it can be anticipated as a new, highly sensitive, and rapid screening method.

Analyte(s)	Sample(s)	Extraction	Clean-up	Determination	Ref.
Imidacloprid	Water	Liquid–liquid extraction with dichloromethane	None	FIA with photochemically induced fluorescence detection	Vílchez et al., 2001
Imidacloprid	Water, hemlock xylem fluid and grape	Only filtration	None	FIA with chemiluminescence detection	Lagalante & Greenbacker, 2007
	Honey	Only dilution			
Imidacloprid	Water	Only filtration	None	FIA with photochemically induced fluorescence detection	Flores et al., 2007
16 pesticides and metabolite including nitenpyram and thiacloprid	Orange, lemon, apple, green pepper, persimmon, grapefruit, tomato, pear and grape	QuEChERS method		Desorption ESI-MS and MS/MS	García-Reyes et al., 2009
		Shaking with acetonitrile	Dispersive PSA SPE		
	Fruir and vegetable skin (peel)	None	None		

Table 2. Overview of nonchromatographic techniques with FIA and MS for determination of neonicotinoid insecticides.

3.2 ELISA analysis for neonicotinoid insecticides as a rapid and simple preliminary screening method

Since Yalow and Berson applied radioimmunoassay (RIA) to the determination of insulin in the 1950s (Yalow & Berson, 1959, 1960), RIA has been widely used mainly in the field of clinical laboratory tests. Although immunoassay for pesticide determination was not reported in two decades. Subsequently, antibodies selective to DDT and malathion were developed (Centeno et al., 1970). In addition, RIA for parathion determination was developed (Ercegovich et al., 1981). After Engvall and Perlmann (1972) proposed enzyme immunoassay (EIA, ELISA) using enzyme-labeled antigens instead of radioisotope-labeled antigens, a marked increase existed in the development of ELISA for various pesticides including organophosphorus and synthetic pyrethroid insecticides (Hennion et al., 1998; Meulenberg et al., 1995; Nunes et al., 1998; Shan et al., 2002).

The immunogenicity of small molecules such as pesticides themselves is extremely low. Therefore, it is necessary for the development of antigens to these compounds to design and synthesize hapten molecules that imitate the chemical structure of the target substances. It is known that the measurement sensitivity and selectivity of the resulting antibody is strongly dependent on the chemical structure of the designed hapten molecule (Shan et al., 2002; Szurdoki et al., 1995). However, the importance of hapten design is not explained in this chapter.

ELISA, based on an antigen–antibody reaction, is a method used to detect residual pesticides, etc., in various samples consisting of complex matrices such as food samples including crops. Therefore, fast analysis can be achieved because significant laborsaving is possible in complicated sample pre-treatment procedures before chromatographic determination. Moreover, ELISA is regarded as an economical, straightforward, and easy analytical method because only small amounts of organic solvents are used, instruments requiring expertise are not needed, and multisample treatment is possible using 96-well microplates (Ellis, 1996). In contrast, ELISA is disadvantageous compared to chromatographic determination in that it is limited to the determination of a single pesticide, it is incapable of identification, and it might produce a false positive result when it cross-reacts to a compound with similar chemical structure because it is a selective analytical method.

As shown in Table 3, ELISA for the determination of neonicotinoid insecticides was first developed in 2000 using polyclonal antibody (PoAb) specific to imidacloprid (Li & Li, 2000). It was followed by a report by Lee et al. (2001) in which the measurement sensitivity was improved to be approximately twice that obtained using another hapten. Watanabe et al. (2001) and Kim et al. (2004) developed monoclonal antibody (MoAb) specific to imidacloprid, and constructed ELISA that is 5-fold to 20-fold more sensitive than that obtained using PoAb. In addition to imidacloprid, ELISA using MoAb specific to acetamiprid (Watanabe et al., 2001) and thiamethoxam (Kim et al., 2003, 2006) were also developed.

As described above, ELISA uses highly specific antigen–antibody reaction. Theoretically, it responds sensitively only to the trace pesticide in matrices. In fact, however, ELISA is susceptible to the matrix effect described in Section 2.2, and it is important to evaluate the matrix effect for individual measurement samples (Jourdan et al., 1996; Nunes et al., 1998; Skerritt & Rani, 1996). While the matrix effect can be avoided or reduced by SPE, etc., such measures would eliminate advantages of ELISA shown above. Table 3 shows that the easiest method to avoid the matrix effect is dilution of the sample extract (mainly methanol extract)

with water or phosphate buffer. The recovery from various measurement samples were generally good in all reports (Byrne et al., 2005; Eisenback et al., 2009; Kim et al., 2006; Ma et al., 2009; Watanabe et al., 2001; Watanabe et al., 2004a, 2004b, 2006, 2007, 2011; Xu et al., 2010). The measurement sensitivity of ELISA is apt to be affected by the concentration of extraction solvent (mainly methanol) coexisting in the sample solution during measurement (Nunes et al., 1998). The sample extraction is diluted not only to avoid the matrix effect effectively but also to reduce the influence of the organic solvent.

Recently, kit-based ELISA for neonicotinoid insecticides was developed and marketed by Horiba Ltd. (Kyoto, Japan) and Envirologix Inc. (Portland, ME). Kit-based ELISA package reagents needed for determination (96-well microplate pre-coated with antibody, washing solution, substrate solution, and stopping solution, etc.) can be used easily for monitoring tests of a specific neonicotinoid insecticide. However, it is important in the use of kit-based ELISA to remember that the matrix effect should be evaluated in advance (Byrne et al., 2005; Watanabe et al., 2004a, 2004b, 2006, 2007, 2011).

In any event, when ELISA is applied to a sample, it might be affected by the matrix effect. Therefore, it is important to examine in advance if the matrix effect is present, and to examine methods to avoid it. At least, ELISA can be applied sufficiently to routine analysis, especially as a screening method, by solving this problem.

Analyte(s)	Sample(s)	Extraction	Following sample preparation	Antibody	Assay format	IC_{50}	Ref.
Imidacloprid	Water	None	None	PoAb	Indirect competitive ELISA	35 ppb	Li & Li, 2000
	Coffee cherry and coffee bean	Homogenization with methanol/1% sulfuric acid (3:1, v/v)	Evaporation, extraction with ethyl acetate and reconstitution with buffer				
Imidacloprid	Apple	Homogenization with methanol	Evaporation and reconstitution with buffer	PoAb	Indirect competitive ELISA	17.3 ng/mL	Lee et al., 2001
	Water	None	Dilution with buffer				
		Extraction with ethyl acetate from C_{18} SPE	Evaporation and reconstitution with buffer				
Acetamiprid	Cucumber, green pepper, tomato and apple	Shaking with methanol	Centrifugation and dilution with methanol/buffer (9:1, v/v)	MoAb	Direct competitive ELISA	1.0 ng/mL	Watanabe et al., 2001
Imidacloprid				MoAb		3.3 ng/mL	
Thiamethoxam	Water	None	Diluted with buffer	PoAb	Direct competitive ELISA	9.0 ng/mL	Kim et al., 2003
Imidacloprid	Apple	Shaking by hand with methanol	Filtration and dilution with water	MoAb	Direct competitive ELISA kit	8 ng/g	Watanabe et al., 2004a
Imidacloprid	Cucumber, eggplant, lettuce, green pepper and spinach	Shaking by hand with methanol	Filtration and dilution with water	MoAb	Direct competitive ELISA kit	-	Watanabe et al., 2004b
		Ultrasonic extraction with methanol					

Imidacloprid	Water	None	Dilution with buffer	MoAb	Indirect competitive ELISA	0.8 μg/L	Kim et al., 2004
	Cucumber	Extraction with methanol	Centrifugation, filtration and dilution with buffer				
Imidacloprid	Avocado leaf	Homogenization with water	Dilution with water	-	Direct competitive ELISA kit	-	Byrne et al., 2005
Imidacloprid	Wiliwili leaf	Ultrasonic extraction with methanol/0.04% sulfuric acid (4:1, v/v)	Filtration, evaporation, centrifugation, liquid–liquid partition, reconstitution and dilution with water	MoAb	Indirect competitive ELISA	6.82 ppb	Xu et al., 2006
Thiamethoxam	Water	None	Filtration and dilution with buffer	MoAb	Flow fluorescent immunoassay	30 pg/mL	Kim et al., 2006
	Potato, cucumber and apple	Shaking with methanol/water (7:3, v/v)	Centrifugation, filtration and dilution with buffer				
Acetamiprid	Peach, apple, strawberry, cucumber, eggplant and tomato	Vortex mixing with methanol	Filtration and dilution with water	MoAb	Direct competitive ELISA kit	0.6 ng/g	Watanabe et al., 2006
Imidacloprid	Apple juice, grape juice and orange juice	None	Dilution with buffer	MoAb	Direct competitive ELISA kit	3.9 μg/L	Watanabe et al., 2007
Imidacloprid	Hemlock wood and needle tissues	Shaking by hand with water	Centrifugation and dilution with water	-	Direct competitive ELISA kit	-	Eisenback et al., 2009
Imidacloprid	Honey	None	Dilution with buffer	MoAb	Indirect competitive ELISA	6.5 ng/mL	Ma et al., 2009
Thiamethoxam						0.57 ng/mL	
Imidacloprid	Apple juice, grape juice, orange juice and peach juice	None	Dilution with buffer	MoAb	Indirect competitive ELISA	6.2 ng/mL	Xu et al., 2010
Thiamethoxam						0.5 ng/mL	
Imidacloprid	Honeybee	Liquid–liquid extraction with dichloromethane after homogenization with acetone and coagulation	Dilution with water	PoAb	Indirect competitive chemiluminescent ELISA	14.8 ng/mL	Girotti et al., 2010
Imidaclothiz	Water	None	Dilution with methanol/buffer (2:8, v/v)	MoAb	Indirect competitive ELISA	0.0875 mg/L	Fang et al., 2011
	Soil and cabbage	Ultrasonic extraction with	Centrifugation, evaporation and				

		dichloromethane	reconstitution with methanol/buffer (1:9, v/v)				
Dinotefuran	Rice	Mechanical shaking with methanol	Centrifugation and dilution with water (if necessary, dilution with methanol/water (1:9, v/v))	MoAb	Direct competitive ELISA kit	5.4 ng/mL	Watanabe et al., 2011

Table 3. Overview of nonchromatographic techniques with immunochemical determinations for determination of neonicotinoid insecticides.

4. Overview of analytical methods for pesticide residues including neonicotinoid insecticides

Sections 2 and 3 summarize trends in the determination methods for neonicotinoid insecticides and the problems discovered during the development of analytical methods such as the matrix effect. At present, highly accurate and sensitive multiresidue analysis by GC or HPLC equipped with MS or MS/MS as a detector is the major trend in the determination not only of neonicotinoid insecticides but also of pesticides in general. It goes without saying that pesticide residue analysis is an indispensable basic technology that is useful to secure the safety of food, including crops, as well as in various research fields such as environment and ecological impact evaluation. However, it is questionable because it is entirely dependent only on GC–MS, LC-MS, and LC-MS/MS for pesticide residue analysis. Particularly when pesticide residues are analyzed to secure food safety, two situations are assumed: (1) testing of foods on the market, and (2) testing of crops before shipment. In the former situation, the history of pesticides that have been used is often unknown. Therefore multiresidue analysis using MS or MS/MS is suitable, in which as many pesticides as possible can be tested and unknown ingredients can be identified. On the other hand, in the latter situation, the subject pesticide can be selected based on the history of use. Therefore there might be cases in which it is more sensible to choose a rapid, simple, and easy method such as ELISA described in Section 3 over multiresidue analysis which has better than necessary performance.

In any event, among the numerous analytical methods, it is necessary to make a wise choice for a suitable method via accurate comprehension of the analytical objectives.

5. References

Agüera, A.; López, S.; Fernández-Alba, A.R.; Contreras, M.; Crespo, J. & Piedra, L. (2004). One-year routine application of a new method based on liquid chromatography – tandem mass spectrometry to the analysis of 16 multiclass pesticides in vegetable samples. *Journal of Chromatography A*, Vol. 1045, No. 1-2, (August 2004), pp. 125-135, ISSN 0021-9673

Anastassiades, M.; Lehotay, S.J.; Štajnbaher, D. & Schenck, F.J. (2003). Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "Dispersive solid-phase extraction" for the determination of pesticide residues in produce. *Journal of AOAC International*, Vol. 86, No. 2, (March 2003), pp. 412-431, ISSN 1060-3271

Barker, S.A.; Long, A.R. & Short, C.R. (1989). Isolation of drug residues from tissues by solid phase dispersion. *Journal of Chromatography A*, Vol. 475, No. 2, (1989), pp. 353-361, ISSN 0021-9673

Barker, S.A. (2000a). Applications of matrix solid-phase dispersion in food analysis. *Journal of Chromatography A*, Vol. 880, No. 1-2, (June 2000), pp. 63-68, ISSN 0021-9673

Barker, S.A. (2000b). Matrix solid-phase dispersion. *Journal of Chromatography A*, Vol. 885, No. 1-2, (July 2000), pp. 115-127, ISSN 0021-9673

Baskaran, S.; Kookana, R.S. & Naidu, R. (1997). Determination of the insecticide imidacloprid in water and soil using high-performance liquid chromatography. *Journal of Chromatography A*, Vol. 787, No. 1-2, (November 1997), pp. 271-275, ISSN 0021-9673

Blasco, C.; Font, G. & Picó, Y. (2002a). Comparison of microextraction procedures to determine pesticides in oranges by liquid chromatography – mass spectrometry. *Journal of Chromatography A*, Vol. 970, No. 1-2, (September 2002), pp. 201-212, ISSN 0021-9673

Blasco, C.; Fernández, M.; Picó, Y.; Font, G. & Mañes, J. (2002b). Simultaneous determination of imidacloprid, carbendazim, methiocarb and hexythiazox in peaches and nectarines by liquid chromatography – mass spectrometry. *Analytica Chimica Acta*, Vol. 461, No. 1, (June 2002), pp. 109-116, ISSN 0003-2670

Bonmatin, J.M.; Moineau, I.; Charvet, R.; Fleche, C.; Colin, M.E. & Bengsch, E.R. (2003). A LC/APCI-MS/MS method for analysis of imidacloprid in soils, in plants, and in pollens. *Analytical Chemistry*, Vol. 75, No. 9, (May 2003), pp. 2027-2033, ISSN 0003-2700

Bourgin, M.; Bize, M.; Durand, S.; Albet, J. & Violleau, F. (2009). Development of a rapid determination of pesticides in coated seeds using a high-performance liquid chromatography – UV detection system. *Journal of Agricultural and Food Chemistry*, Vol. 57, No. 21, (November 2009), pp. 10032-10037, ISSN 0021-8561

Byrne, F.J.; Toscano, N.C.; Urena, A.A. & Morse, J.G. (2005). Quantification of imidacloprid toxicity to avocado thrips, *Scirtothrips perseae* Nakahara (Thysanoptera: Thripidae), using a combined bioassay and ELISA approach. *Pest Management Science*, Vol. 61, No. 8, (August 2005), pp. 754-758, ISSN 1526-498X

Carretero, A.S.; Cruces-Blanco, C.; Durán, S.P. & Gutiérrez, A.F. (2003). Determination of imidacloprid and its metabolite 6-chloronicotinic acid in greenhouse air by application of micellar electrokinetic capillary chromatography with solid-phase extraction. *Journal of Chromatography A*, Vol. 1003, No. 1-2, (June 2003), pp. 189-195, ISSN 0021-9673

Centeno, E.R.; Johnson, W.J. & Sehon, A.H. (1970). Antibodies to two common pesticides, DDT and malathion, *International Archives of Allergy and Applied Immunology*, Vol. 37, No. 1, (1970), pp.1-13, ISSN 1018-2438

Chin-Chen, M-L.; Esteve-Romero, J. & Carda-Broch, S. (2009). Determination of the insecticide imidacloprid in fruit juices using micellar high-performance liquid chromatography. *Journal of AOAC International*, Vol. 92, No. 5, (October 2009), pp. 1551-1556, ISSN 1060-3271

Decourtye, A. & Devillers, J. (2010). Ecotoxicity of neonicotinoid insecticides to bees, In: *Insect Nicotinic Acetylcholine Receptors*, *Advances in Experimental Medicine and Biology*, Vol. 683, S.H. Thany, pp. 85-95, Springer, ISBN 978-1441964441, New York

de Erenchun, N.R.; de Balugera, Z.G.; Goicolea, M.A. & Barrio, R.J. (1997). Determination of imidacloprid and its major metabolite in soils by liquid chromatography with pulsed reductive amperometric detection. *Analytica Chimica Acta*, Vol. 349, No. 1-3, (August 1997), pp. 199-206, ISSN 0003-2670

Di Muccio, A.; Fidente, P.; Barbini, D.A.; Dommarco, R.; Seccia, S. & Morrica, P. (2006). Application of solid-phase extraction and liquid chromatography – mass spectrometry to the determination of neonicotinoid pesticide residues in fruit and vegetables. *Journal of Chromatography A*, Vol. 1108, No. 1, (March 2006), pp. 1-6, ISSN 0021-9673

Economou, A.; Botitsi, H.; Antoniou, S. & Tsipi, D. (2009). Determination of multi-class pesticides in wines by solid-phase extraction and liquid chromatography – tandem mass spectrometry. *Journal of Chromatography A*, Vol. 1216, No. 31, (July 2009), pp. 5856-5867, ISSN 0021-9673

Eisenback, B.M.; Mullins, D.E.; Salom, S.M. & Kok, L.T. (2008). Evaluation of ELISA for imidacloprid detection in eastern hemlock (*Tsuga Canadensis*) wood and needle tissues. *Pest Management Science*, Vol. 65, No. 2, (February 2009), pp. 122-128, ISSN 1526-498X

El Hassani, A.K.; Dacher, M.; Gary, V.; Lambin, M.; Gauthier, M. & Armengaud, C. (2008). Effects of sublethal doses of acetamiprid and thiamethoxam on the behavior of the honeybee (*Apis mellifera*). *Archives of Environmental Contamination and Toxicology*, Vol. 54, No. 4, (May 2008), pp. 653-661, ISSN 0090-4341

Ellis, R.L. (1996). Rapid test methods for regulatory programs, In: *Immunoassays for Residue Analysis, Food Safety*, R.C. Beier & L.H. Stanker (ACS Symposium Series 621), pp. 44-58, American Chemical Society, ISBN 0-8412-3379-9, Washington, DC

Engvall, E. & Perlmann, P. (1972). Enzyme-linked immunosorbent assay, Elisa: III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. *Journal of Immunology*, Vol. 109, No. 1, (July 1972), pp. 129-135, ISSN 0022-1767

Ercegovich, C.D.; Vallejo, R.P.; Gettig, R.R.; Woods, L.; Bogus, E.R. & Mumma, R.O. (1981). Development of a radioimmunoassay for parathion, *Journal of Agricultural and Food Chemistry*, Vol. 29, No. 3, (May 1981), pp. 559-563, ISSN 0021-8561

Fang, S.; Zhang, B.; Ren, K.; Cao, M.; Shi, H. & Wang, M. (2011). Development of a sensitive indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) based on the monoclonal antibody for the detection of the imidaclothiz residue. *Journal of Agricultural and Food Chemistry*, Vol. 59, No. 5, (February 2011), pp. 1594-1597, ISSN 0021-8561

Fernandez-Alba, A.R.; Valverde, A.; Agüera, A.; Contreras, M. & Chiron, S. (1996). Determination of imidacloprid in vegetables by high-performance liquid chromatography with diode-array detection. *Journal of Chromatography A*, Vol. 721, No. 1, (January 1996), pp. 97-105, ISSN 0021-9673

Fernández-Alba, A.R.; Tejedor, A.; Agüera, A.; Contreras, M. & Garrido, J. (2000). Determination of imidacloprid and benzimidazole residues in fruits and vegetables by liquid chromatography – mass spectrometry after ethyl acetate multiresidue extraction. *Journal of AOAC International*, Vol. 83, No. 3, (May 2000), pp. 748-755, ISSN 1060-3271

Ferrer, I.; Thurman, E.M. & Fernández-Alba, A.R. (2005). Quantitation and accurate mass analysis of pesticides in vegetables by LC/TOF-MS. *Analytical Chemistry*, Vol. 77, No. 9, (May 2005), pp. 2818-2825, ISSN 0003-2700

Ferrer, I. & Thurman, E.M. (2007). Multi-residue method for the analysis of 101 pesticides and their degradates in food and water samples by liquid chromatography/time-of-flight mass spectrometry. *Journal of Chromatography A*, Vol. 1175, No. 1, (December 2007), pp. 24-37, ISSN 0021-9673

Fidente, P.; Seccia, S.; Vanni, F. & Morrica, P. (2005). Analysis of nicotinoid insecticides residues in honey by solid matrix partition clean-up and liquid chromatography – electrospray mass spectrometry. *Journal of Chromatography A*, Vol. 1094, No. 1-2, (November 2005), pp. 175-178, ISSN 0021-9673

Flores, J.L.; Díaz, A.M. & de Córdove, M.L.F. (2007). Development of a photochemically induced fluorescence-based optosensor for the determination of imidacloprid in peppers and environmental waters. *Talanta*, Vol. 72, No. 3, (May 2007), pp. 991-991, ISSN 0039-9140

Frenich, A.G.; González, F.J.E.; Vidal, J.L.M.; Vázquez, P.P. & Sánchez, M.M. (2000). Determination of imidacloprid and its metabolite 6-chloronicotinic acid in greenhouse air by high-performance liquid chromatography with diode-array detection. *Journal of Chromatography A*, Vol. 869, No. 1-2, (February 2000), pp. 497-504, ISSN 0021-9673

Frenich, A.G.; Vidal, J.L.M.; Pastor-Montoro, E. & Romero-González, R. (2008). High-throughput determination of pesticide residues in food commodities utilizing ultra-performance liquid chromatography – tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*, Vol. 390, No. 3, (February 2008), pp. 947-959, ISSN 1618-2642

Fritz, J.S. & Macka, M. (2000). Solid-phase trapping of solutes for further chromatographic or electrophoretic analysis. *Journal of Chromatography A*, Vol. 902, No. 1, (November 2000), pp. 137-166, ISSN 0021-9673

Galera, M.M.; Frenich, A.G.; Vidal, J.L.M. & Vázquez, P.P. (1998). Resolution of imidacloprid pesticide and its metabolite 6-chloronicotinic acid using cross-sections of spectrochromatograms obtained by high-performance liquid chromatography with diode-array detection. *Journal of Chromatography A*, Vol. 799, No. 1-2, (March 1998), pp. 149-154, ISSN 0021-9673

García, M.D.G.; Galera, M.M.; Valverde, R.S.; Galanti, A. & Girotti, S. (2007). Column switching liquid chromatography and post-column photochemically fluorescence detection to determine imidacloprid and 6-chloronicotinic acid in honeybees. *Journal of Chromatography A*, Vol. 1147, No. 1, (April 2007), pp. 17-23, ISSN 0021-9673

García-Chao, M.; Agruña, M.J.; Calvete, G.F.; Sakkas, V.; Llompart, M. & Dagnac, T. (2010). Validation of an off line solid phase extraction liquid chromatography – tandem mass spectrometry method for the determination of systemic insecticide residues in honey and pollen samples collected in apiaries from NW Spain. *Analytica Chimica Acta*, Vol. 672, No. 1-2, (July 2010), pp. 107-113, ISSN 0003-2670

García-Reyes, J.F.; Jackson, A.U.; Molina-Díaz, A. & Cooks, R.G. (2009). Desorption electrospray ionization mass spectrometry for trace analysis of agrochemicals in Food. *Analytical Chemistry*, Vol. 81, No. 2, (January 2009), pp. 820-829, ISSN 0003-2700

Girolami, V.; Mazzon, L.; Squartini, A.; Mori, N.; Marzaro, M.; Di Bernardo, A.; Greatti, M.; Giorio, C. & Tapparo, A. (2009). Translocation of neonicotinoid insecticides from coated seeds to seedling guttation drops: A novel mode of intoxication for bees. *Journal of Economic Entomology*, Vol. 102, No. 5, (October 2009), pp. 1808-1815, ISSN 0022-0493

Girotti, S.; Maiolini, E.; Ghini, S.; Eremin, S. & Mañes, J. (2010). Quantification of imidacloprid in honeybees: Development of a chemiluminescent ELISA. *Analytical Letters*, Vol. 43, No. 3, (2010), pp. 466-475, ISSN 0003-2719

Hengel, M.J. & Miller, M. (2008). Analysis of pesticides in dried hops by liquid chromatography – tandem mass spectrometry. *Journal of Agricultural and Food Chemistry*, Vol. 56, No. 16, (August 2008), pp. 6851-6856, ISSN 0021-8561

Hennion, M-C. & Barcelo, D. (1998). Strengths and limitations of immunoassays for effective and efficient use for pesticide analysis in water samples: A review. *Analytica Chimica Acta*, Vol. 362, No. 1, (April 1998), pp.3-34, ISSN 0003-2670

Hernández, F.; Pozo, O.J.; Sancho, J.V.; Bijlsma, L.; Barreda, M. & Pitarch, E. (2006). Multiresidue liquid chromatography tandem mass spectrometry determination of 52 non gas chromatography-amenable pesticides and metabolites in different food commodities. *Journal of Chromatography A*, Vol. 1109, No. 2, (March 2006), pp. 242-252, ISSN 0021-9673

Ishii, Y.; Kobori, I.; Araki, Y.; Kurogochi, S.; Iwaya, K. & Kagabu, S. (1994). HPLC determination of the new insecticide imidacloprid and its behavior in rice and cucumber. *Journal of Agricultural and Food Chemistry*, Vol. 42, No. 12, (December 1994), pp. 2917-2921, ISSN 0021-8561

Iwasa, T.; Motoyama, N.; Ambrose, J.T. & Roe, R.M. (2004). Mechanism for the differential toxicity of neonicotiniod insecticides in the honey bee, *Apis mellifera*. *Crop Protection*, Vol. 23, No. 5, (May 2004), pp.371-378, ISSN 0261-2194

Jansson, C.; Pihlström, T.; Österdahl, B-G. & Markides, K.E. (2004). A new multi-residue method for analysis of pesticide residues in fruit and vegetables using liquid chromatography with tandem mass spectrometric detection. *Journal of Chromatography A*, Vol. 1023, No. 1, (January 2004), pp. 93-104, ISSN 0021-9673

Jourdan, S.W.; Scutellaro, A.M.; Hayes, M.C. & Herzog, D.P. (1996). Adapting immunoassays to the analysis of food samples, In: *Immunoassays for Residue Analysis, Food Safety*, R.C. Beier & L.H. Stanker (ACS Symposium Series 621), pp. 17-28, American Chemical Society, ISBN 0-8412-3379-9, Washington, DC

Kagabu, S. (1996). Studies on the synthesis and insecticidal activity of neonicotinoid compounds. *Journal of Pesticide Science*, Vol. 21, No. 2, (May 1996), pp. 231-239, ISSN 1348-589X

Kamel, A. (2010). Refined methodology for the determination of neonicotinoid pesticides and their metabolites in honey bees and bee products by liquid chromatography – tandem mass spectrometry (LC-MS/MS). *Journal of Agricultural and Food Chemistry*, Vol. 58, No. 10, (May 2010), pp. 5926-5931, ISSN 0021-8561

Kamel, A.; Qian, Y.; Kolbe, E. & Stafford, C. (2010). Development and validation of a multiresidue method for the determination of neonicotinoid and macrocyclic lactone pesticide residues in milk, fruits, and vegetables by ultra-performance liquid chromatography/MS/MS. *Journal of AOAC International*, Vol. 93, No. 2, (April 2009), pp. 389-399, ISSN 1060-3271

Kanrar, B.; Mandal, S. & Bhattacharyya, A. (2010). Validation and uncertainty analysis of a multiresidue method for 42 pesticides in made tea, tea infusion and spent leaves using ethyl acetate extraction and liquid chromatography – tandem mass spectrometry. *Journal of Chromatography A*, Vol. 1217, No. 12, (March 2010), pp. 1926-1933, ISSN 0021-9673

Kim, H.-J.; Liu, S.; Keum, Y.-S. & Li, Q.X. (2003). Development of an enzyme-linked immunosorbent assay for the insecticide thiamethoxam. *Journal of Agricultural and Food Chemistry*, Vol. 51, No. 7, (March 2003), pp. 1823-1830, ISSN 0021-8561

Kim, H.-J.; Shelver, W.L. & Li, Q.X. (2004). Monoclonal antibody-based enzyme-linked immunosorbent assay for the insecticide imidacloprid. *Analytica Chimica Acta*, Vol. 509, No. 1, (April 2004), pp. 111-118, ISSN 0003-2670

Kim, H.-J.; Shelver, W.L.; Hwang, E.-C.; Xu, T. & Li, Q.X. (2006). Automated flow fluorescent immunoassay for part per trillion detection of the neonicotinoid insecticide thiamethoxam. *Analytica Chimica Acta*, Vol. 571, No. 1, (June 2006), pp. 66-73, ISSN 0003-2670

Kmellár, B.; Fodor, P.; Pareja, L.; Ferrer, C.; Martínez-Uroz, M.A.; Valverde, A. & Fernandez-Alba, A.R. (2008). Validation and uncertainty study of a comprehensive list of 160 pesticide residues in multi-class vegetables by liquid chromatography – tandem mass spectrometry. *Journal of Chromatography A*, Vol. 1215, No. 1-2, (December 2008), pp. 37-50, ISSN 0021-9673

Kmellár, B.; Pareja, L.; Ferrer, C.; Fodor, P. & Fernandez-Alba, A.R. (2011). Study of the effects of operational parameters on multiresidue pesticide analysis by LC-MS/MS. *Talanta*, Vol. 84, No. 2, (April 2011), pp. 262-273, ISSN 0039-9140

Lagalante, A.F. & Greenbacker, P.W. (2007). Flow injection analysis of imidacloprid in natural waters and agricultural matrixes by photochemical dissociation, chemical reduction, and nitric oxide chemiluminescence detection. *Analytica Chimica Acta*, Vol. 590, No. 2, (May 2007), pp. 151-158, ISSN 0003-2670

Lara, F.J.; García-Campaña, A.M. & Aaron, J-J. (2010). Analytical applications of photoinduced chemiluminescence in flow systems - A review. *Analytica Chimica Acta*, Vol. 679, No. 1-2, (October 2010), pp. 17-30, ISSN 0003-2670

Leandro, C.C.; Hancock, P.; Fussell, R.J. & Keely, B.J. (2007). Ultra-performance liquid chromatography for the determination of pesticide residues in foods by tandem quadrupole mass spectrometry with polarity switching. *Journal of Chromatography A*, Vol. 1144, No. 2, (March 2007), pp. 161-169, ISSN 0021-9673

Lee, J.K.; Ahn, K.C.; Park, O.S.; Kang, S.Y. & Hammock, B.D. (2001). Development of an ELISA for detection of the residues of the insecticide imidacloprid in agricultural and environmental samples. *Journal of Agricultural and Food Chemistry*, Vol. 49, No. 5, (May 2001), pp. 2159-2167, ISSN 0021-8561

Lee, S.M. & Wylie, P.L. (1991). Comparison of the atomic emission detector to other element-selective detectors for the gas chromatographic analysis of pesticide residues. *Journal of Agricultural and Food Chemistry*, Vol. 39, No. 12, (December 1991), pp. 2192-2199, ISSN 0021-8561

Lehotay, S.J.; de Kok, A.; Hiemstra, M. & van Bodegraven, P. (2005). Validation of a fast and easy method for the determination of residues from 229 pesticides in fruits and vegetables using gas and liquid chromatography and mass spectrometric detection.

Journal of AOAC International, Vol. 88, No. 2, (March 2005), pp. 595-614, ISSN 1060-3271

Li, K. & Li, Q.X. (2000). Development of an enzyme-linked immunosorbent assay for the insecticide imidacloprid. *Journal of Agricultural and Food Chemistry*, Vol. 48, No. 8, (August 2000), pp. 3378-3382, ISSN 0021-8561

Liu, H.; Song, J.; Zhang, S.; Qu, L.; Zhao, Y.; Wu, Y. & Liu, H. (2005). Analysis of residues of imidacloprid in tobacco by high-performance liquid chromatography with liquid–liquid partition cleanup. *Pest Management Science*, Vol. 61, No. 5, (May 2005), pp. 511-514, ISSN 1526-498X

Liu, S.; Zheng, Z.; Wei, F.; Ren, Y.; Gui, W.; Wu, H. & Zhu, G. (2010). Simultaneous determination of seven neonicotinoid pesticide residues in food by ultraperformance liquid chromatography tandem mass spectrometry. *Journal of Agricultural and Food Chemistry*, Vol. 58, No. 6, (March 2010), pp. 3271-3278, ISSN 0021-8561

Llorent-Martínez, E.J.; Ortega-Barrales, P.; Fernández-de Córdova, M.L. & Ruiz-Medina, A. (2011). Trends in flow-based analytical methods applied to pesticide detection: A review. *Analytica Chimica Acta*, Vol. 684, No. 1-2, (January 2011), pp. 30-39, ISSN 0003-2670

Ma, H.; Xu, Y.; Li, Q.X.; Xu, T.; Wang, X. & Li, J. (2009). Application of enzyme-linked immunosorbent assay for quantification of the insecticides imidacloprid and thiamethoxam in honey samples. *Food Additives and Contaminants*, Vol. 26, No. 5, (May 2009), pp. 713-718, ISSN 1944-0049

MacDonald, L.M. & Meyer, T.R. (1998). Determination of imidacloprid and triadimefon in white pine by gas chromatography/mass spectrometry. *Journal of Agricultural and Food Chemistry*, Vol. 46, No. 8, (August 1998), pp. 3133-3138, ISSN 0021-8561

Mateu-Sánchez, M.; Moreno M.; Arrebola, F.J. & Vidal, J.L.M. (2003). Analysis of acetamiprid in vegetables using gas chromatography – tandem mass spectrometry. *Analytical Sciences*, Vol. 19, No. 5, (May 2003), pp. 701-704, ISSN 0910-6340

Mayer-Helm, B. (2009). Method development for the determination of 52 pesticides in tobacco by liquid chromatography – tandem mass spectrometry. *Journal of Chromatography A*, Vol. 1216, No. 51, (December 2009), pp. 8953-8959, ISSN 0021-9673

Meulenberg, E.P.; Mulder, W.H. & Stoks, P.G. (1995). Immunoassays for pesticides. *Environmental Science and Technology*, Vol. 29, No. 3, (March 1995), pp. 553-561, ISSN 0013-936X

Mohan, C.; Kumar, Y.; Madan, J. & Saxena, N. (2010). Multiresidue analysis of neonicotinoids by solid-phase extraction technique using high-performance liquid chromatography. *Environmental Monitoring and Assessment*, Vol. 165, No. 1-4, (June 2010), pp. 573-576, ISSN 0167-6369

Mommaerts, V.; Reynders, S.; Boulet, J.; Besard, L.; Sterk, G. & Smagghe, G. (2010). Risk assessment for side-effects of neonicotinoids against bumblebees with and without impairing foraging behavior. *Ecotoxicology*, Vol. 19, No. 1, (January 2010), pp. 207-215, ISSN 0963-9292

Nauen, R.; Ebbinghaus-Kintscher, U. & Schmuck, R. (2001). Toxicity and nicotinic acetylcholine receptor interaction of imidacloprid and its metabolites in *Apis*

mellifera (Hymenoptera: Apidae). *Pest Management Science*, Vol. 57, No. 7, (July 2001), pp. 577-586, ISSN 1526-498X

Navalón, A.; González-Casado, A.; El-Khattabi, R.; Vilchez, J.L. & Fernández-Alba, A.R. (1997). Determination of imidacloprid in vegetable samples by gas chromatography – mass spectrometry. *Analyst*, Vol. 122, No. 6, (June 1997), pp. 579-581, ISSN 0003-2654

Niessen, W.M.A.; Manini, P. & Andreoli, R. (2006). Matrix effects in quantitative pesticide analysis using liquid chromatography – mass spectrometry. *Mass Spectrometry Reviews*, Vol. 25, No. 6, (June 2006), pp. 881-899, ISSN 1098-2787

Nunes, G.S.; Toscano, I.A. & Barceló, D. (1998). Analysis of pesticides in food and environmental samples by enzyme-linked immunosorbent assays. *Trends in Analytical Chemistry*, Vol. 17, No. 2, (February 1998), pp. 79-87, ISSN 0165-9936

Obana, H.; Okihashi, M.; Akutsu, K.; Kitagawa, Y. & Hori, S. (2002). Determination of acetamiprid, imidacloprid, and nitenpyram residues in vegetables and fruits by high-performance liquid chromatography with diode-array detection. *Journal of Agricultural and Food Chemistry*, Vol. 50, No. 16, (July 2002), pp. 4464-4467, ISSN 0021-8561

Obana, H.; Okihashi, M.; Akutsu, K.; Kitagawa, Y. & Hori, S. (2003). Determination of neonicotinoid pesticide residues in vegetables and fruits with solid phase extraction and liquid chromatography mass spectrometry. *Journal of Agricultural and Food Chemistry*, Vol. 51, No. 9, (April 2003), pp. 2501-2505, ISSN 0021-8561

Pareja, L.; Martínez-Bueno, M.J.; Cesio, V.; Heinzen, H. & Fernández-Alba, A.R. (2011). Trace analysis of pesticides in paddy field water by direct injection using liquid chromatography-quadrupole-linear ion trap-mass spectrometry. *Journal of Chromatography A*, Vol. 1218, No. 30, (July 2011), pp. 4790-4798, ISSN 0021-9673

Payá, P.; Anastassiades, M.; Mack, D.; Sigalova, I.; Tasdelen, B.; Oliva, J. & Barba, A. (2007). Analysis of pesticide residues using the Quick Easy Cheap Effective Rugged and Safe (QuEChERS) pesticide multiresidue method in combination with gas and liquid chromatography and tandem mass spectrometric detection. *Analytical and Bioanalytical Chemistry*, Vol. 389, No. 6, (November 2007), pp. 1697-1714, ISSN 1618-2642

Pirard, C.; Widart, J.; Nguyen, B.K.; Deleuze, C.; Heudt, L.; Haubruge, E.; De Pauw, E. & Focant, J.-F. (2007). Development and validation of a multi-residue method for pesticide determination in honey using on-column liquid–liquid extraction and liquid chromatography – tandem mass spectrometry. *Journal of Chromatography A*, Vol. 1152, No. 1-2, (June 2007), pp. 116-123, ISSN 0021-9673

Pous, X.; Ruís, M.J.; Picó, Y. & Font, G. (2001). Determination of imidacloprid, metalaxyl, myclobutanil, propham, and thiabendazole in fruits and vegetables by liquid chromatography – atmospheric pressure chemical ionization-mass spectrometry. *Fresenius' Journal of Analytical Chemistry*, Vol. 371, No. 2, (September 2001), pp. 182-189, ISSN 0937-0633

Radišić, M.; Grujić, S.; Vasiljević, T.; Laušević, M. (2009). Determination of selected pesticides in fruit juices by matrix solid-phase dispersion and liquid chromatography – tandem mass spectrometry. Food Chemistry, Vol. 113, No. 2, (March 2009), pp. 712-719, ISSN 0308-8146

Rancan, M.; Rossi, S. & Sabatini, A.G. (2006a). Determination of thiamethoxam residues in honeybees by high performance liquid chromatography with an electrochemical detector and post-column photochemical reactor. *Journal of Chromatography A*, Vol. 1123, No. 1, (August 2006), pp. 60-65, ISSN 0021-9673

Rancan, M.; Sabatini, A.G.; Achilli, G. & Galletti, G.C. (2006b). Determination of imidacloprid and metabolites by liquid chromatography with an electrochemical detector and post column photochemical reactor. *Analytica Chimica Acta*, Vol. 555, No. 1, (January 2006), pp. 20-24, ISSN 0003-2670

Romero-González, R.; Frenich, A.G.; Vidal, J.L.M.; Prestes, O.D. & Grio, S.L. (2011). Simultaneous determination of pesticides, biopesticides and mycotoxins in organic products applying a quick, easy, cheap, effective, rugged and safe extraction procedure and ultra-high performance liquid chromatography – tandem mass spectrometry. *Journal of Chromatography A*, Vol. 1218, No. 11, (March 2011), pp. 1477-1485, ISSN 0021-9673

Sannino, A.; Bolzoni, L. & Bandini, M. (2004). Application of liquid chromatography with electrospray tandem mass spectrometry to the determination of a new generation of pesticides in processed fruits and vegetables. *Journal of Chromatography A*, Vol. 1036, No. 2, (May 2004), pp. 161-169, ISSN 0021-9673

Seccia, S.; Fidente, P.; Barbini, D.A. & Morrica, P. (2005). Multiresidue determination of nicotinoid residues in drinking water by liquid chromatography with electrospray ionization mass spectrometry. *Analytica Chimica Acta*, Vol. 553, No. 1-2, (November 2005), pp. 21-26, ISSN 0003-2670

Seccia, S.; Fidente, P.; Montesano, D. & Morrica, P. (2008). Determination of neonicotinoid insecticides residues in bovine milk samples by solid-phase extraction clean-up and liquid chromatography with diode-array detection. *Journal of Chromatography A*, Vol. 1214, No. 1-2, (December 2008), pp. 115-120, ISSN 0021-9673

Shan, G.; Lipton, C.; Gee, S.J. & Hammock, B.D. (2002). Immunoassay, biosensors and other nonchromatographic methods, In: *Handbook of Residue Analytical Methods for Agrochemicals*, P.W. Lee, pp. 623-679, John Wiley & Sons, Ltd., ISBN 0471-49194-2, Chichester

Shiokawa, K.; Tsuboi, S.; Iwaya, K. & Moriya, K. (1994). Development of a chloronicotinyl insecticide, imidacloprid. *Journal of Pesticide Science*, Vol. 19, No. 4, (November 1994), pp. S209-S217, ISSN 1348-589X

Singh, S.B.; Foster, G.D. & Khan, S.U. (2004). Microwave-assisted extraction for the simultaneous determination of thiamethoxam, imidacloprid, and carbendazim residues in fresh and cooked vegetable samples. *Journal of Agricultural and Food Chemistry*, Vol. 52, No. 1, (January 2004), pp. 105-109, ISSN 0021-8561

Skerritt, J.H. & Rani, B.E.A. (1996). Detection and removal of sample matrix effects in agrochemical immunoassays, In: *Immunoassays for Residue Analysis, Food Safety*, R.C. Beier & L.H. Stanker (ACS Symposium Series 621), pp. 29-43, American Chemical Society, ISBN 0-8412-3379-9, Washington, DC

Soloway, S.B.; Henry, A.C.; Kollmeyer, W.D.; Padgett, W.M.; Powell, J.E.; Roman, S.A.; Tieman, C.H.; Corey, R.A. & Horne, C.A. (1978). Nitromethylene heterocycles as insecticides, In: *Pesticide and Venom Neurotoxicity*, D.L. Shankland, R.M. Hollingworth, T. Smith Jr., pp. 153-158, Plenum, ISBN 978-0306311239, New York

Soloway, S.B.; Henry, A.C.; Kollmeyer, W.D.; Padgett, W.M.; Powell, J.E.; Roman, S.A.; Tieman, C.H.; Corey, R.A. & Horne, C.A. (1979). Nitromethylene insecticides, In: *Advances in Pesticide Science*, H. Geissbüehler, pp. 206-217, Pergamon, ISBN 978-0080239309, Oxford

Szurdoki, F.; Bekheit, H.K.M.; Marco, M-P.; Goodrow, M.H. & Hammock, B.D. (1995). Important factors in hapten design and enzyme-linked immunosorbent assay development, In: *New Frontiers in Agrochemical Immunoassay*, D.A. Kurtz, J.H. Skerritt & L. Stanker, pp. 39-63, AOAC International, ISBN 0-935584-58-7, Arlington, VA

Ting, K.-C.; Zhou, E.G. & Saini, N. (2004). Determination of imidacloprid in fruits and vegetables by liquid chromatography with diode array and nitrogen-specific chemiluminescent detection. *Journal of AOAC International*, Vol. 87, No. 4, (July 2004), pp. 997-1002, ISSN 1060-3271

Tokieda, M.; Ozawa, M.; Kobayashi, S. & Gomyo, T. (1997a). Method to determination of total residues of the insecticide acetamiprid and its metabolites in crops by gas chromatography. *Journal of Pesticide Sciences*, Vol. 22, No. 2, (May 1997), pp. 77-83, ISSN 1348-589X

Tokieda, M.; Iiyoshi, K.; Sugioka, K. & Gomyo, T. (1997b). Residue determination method for the insecticide acetamiprid in crops by gas chromatography. *Journal of Pesticide Sciences*, Vol. 22, No. 2, (May 1997), pp. 129-132, ISSN 1348-589X

Tokieda, M.; Tanaka, T.; Ozawa, M. & Gomyo, T. (1998). High performance liquid chromatographic determination of acetamiprid and its degradation products in soil. *Journal of Pesticide Sciences*, Vol. 23, No. 3, (August 1998), pp. 296-299, ISSN 1348-589X

Tomizawa, M. (1994). Structure–activity relationships of nicotinoids and the related compounds. *Journal of Pesticide Science*, Vol. 19, No. 4, (November 1994), pp. S229-S240, ISSN 1348-589X

Tomizawa, M. & Casida, J.E. (2003). Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. *Annual Review of Entomology*, Vol. 48, (2003), pp.339-364, ISSN 0066-4170

Tomizawa, M. & Casida, J.E. (2005). Neonicotinoid insecticide toxicology: Mechanisms of selective action. *Annual Review of Pharmacology and Toxicology*, Vol. 45, (2005), pp. 247-268, ISSN 0362-1642

Tomlin, C.D.S. (2003). *A World Compendium The Pesticide Manual* (Thirteenth Edition), British Crop Protection Council, ISBN 1-901396-13-4, Hampshire, UK

Totti, S.; Fernández, M.; Ghini, S.; Picó, Y.; Fini, F.; Mañes, J. & Girotti, S. (2006). Application of matrix solid phase dispersion to the determination of imidacloprid, carbaryl, aldicarb, and their main metabolites in honeybees by liquid chromatography – mass spectrometry detection. *Talanta*, Vol. 69, No. 3, (May 2006), pp. 724-729, ISSN 0039-9140

Venkateswarlu, P.; Mohan, K.R.; Kumar, C.R. & Seshaiah, K. (2007). Monitoring of multi-class pesticide residues in fresh grape samples using liquid chromatography with electrospray tandem mass spectrometry. *Food Chemistry*, Vol. 105, No. 4, (2007), pp. 1760-1766, ISSN 0308-8146

Vilchez, J.L.; El-Khattabi, R.; Fernández, J.; González-Casado, A. & Navalón, A. (1996). Determination of imidacloprid in water and soil samples by gas chromatography –

mass spectrometry. *Journal of Chromatography A*, Vol. 746, No. 2, (October 1996), pp. 289-294, ISSN 0021-9673

Vilchez, J.L.; Valencia, M.C.; Navalón, A.; Molinero-Morales, B. & Capitán-Vallvey, L.F. (2001). Flow injection analysis of the insecticide imidacloprid in water samples with photochemically induced fluorescence detection. *Analytica Chimica Acta*, Vol. 439, No. 2, (July 2001), pp. 299-305, ISSN 0003-2670

Wan, H.B. & Wong, M.K. (1996). Minimization of solvent consumption in pesticide residue analysis. *Journal of Chromatography A*, Vol. 754, No. 1-2, (November 1996), pp. 43-47, ISSN 0021-9673

Watanabe, E.; Eun, H.; Baba, K.; Arao, T.; Ishii, Y.; Endo, S. & Ueji, M. (2004a). Evaluation and validation of a commercially available enzyme-linked immunosorbent assay for the neonicotinoid insecticide imidacloprid in agricultural samples. *Journal of Agricultural and Food Chemistry*, Vol. 52, No. 10, (May 2004), pp. 2756-2762, ISSN 0021-8561

Watanabe, E.; Eun, H.; Baba, K.; Arao, T.; Ishii, Y.; Endo, S. & Ueji, M. (2004b). Rapid and simple screening analysis for residual imidacloprid in agricultural products with commercially available ELISA. *Analytica Chimica Acta*, Vol. 521, No. 1, (September 2004), pp. 45-51, ISSN 0003-2670

Watanabe, E.; Miyake, S.; Baba, K.; Eun, H. & Endo, S. (2006). Immunoassay for acetamiprid detection: application to residue analysis and comparison with liquid chromatography. *Analytical and Bioanalytical Chemistry*, Vol. 386, No. 5, (November 2006), pp. 1441-1448, ISSN 1618-2642

Watanabe, E.; Baba, K. & Eun, H. (2007). Simultaneous determination of neonicotinoid insecticides in agricultural samples by solid-phase extraction cleanup and liquid chromatography equipped with diode-array detection. *Journal of Agricultural and Food Chemistry*, Vol. 55, No. 10, (May 2007), pp. 3798-3804, ISSN 0021-8561

Watanabe, E.; Baba, K.; Eun, H. & Miyake, S. (2007). Application of a commercial immunoassay to the direct determination of insecticide imidacloprid in fruit juices. *Food Chemistry*, Vol. 102, No. 3, (2007), pp. 745-750, ISSN 0308-8146

Watanabe, E.; Baba, K. & Miyake, S. (2011). Analytical evaluation of enzyme-linked immunosorbent assay for neonicotinoid dinotefuran for potential application to quick and simple screening method in rice samples. *Talanta*, Vol. 84, No. 4, (May 2011), pp. 1107-1111, ISSN 0039-9140

Watanabe, S.; Ito, S.; Kamata, Y.; Omoda, N.; Yamazaki, T.; Munakata, H.; Kaneko, T. & Yuasa, Y. (2001). Development of competitive enzyme-linked immunosorbent assays (ELISAs) based on monoclonal antibodies for chloronicotinoid insecticides imidacloprid and acetamiprid. *Analytica Chimica Acta*, Vol. 427, No. 2, (January 2001), pp. 211-219, ISSN 0003-2670

Xiao, Z.; Li, X.; Wang, X.; Shen, J. & Ding, S. (2011). Determination of neonicotinoid insecticides residues in bovine tissues by pressurized solvent extraction and liquid chromatography – tandem mass spectrometry. *Journal of Chromatography B*, Vol. 879, No. 1, (January 2011), pp. 117-122, ISSN 1570-0232

Xie, W.; Han, C.; Qian, Y.; Ding, H.; Chen, X. & Xi, J. (2011). Determination of neonicotinoid pesticides residues in agricultural samples by solid-phase extraction combined with liquid chromatography – tandem mass spectrometry. *Journal of Chromatography A*, Vol. 1218, No. 28, (July 2011), pp. 4426-4433, ISSN 0021-9673

Xu, T.; Jacobsen, C.M.; Cho, I.K.; Hara, A.H. & Li, Q.X. (2006). Application of an enzyme-linked immunosorbent assay for the analysis of imidacloprid in wiliwili tree, *Erythrina sandwicensis* O. Deg, for control the wasp *Quadrastichus erythrinae*. *Journal of Agricultural and Food Chemistry*, Vol. 54, No. 22, (November 2006), pp. 8444-8449, ISSN 0021-8561

Xu, T.; Wei, K.-Y.; Wang, J.; Ma, H.-X.; Li, J.; Xu, Y.-J. & Li, Q.X. (2010). Quantitative analysis of the neonicotinoid insecticides imidacloprid and thiamethoxam in fruit juices by enzyme-linked immunosorbent assays. *Journal of AOAC International*, Vol. 93, No. 1, (January 2010), pp. 12-18, ISSN 1060-3271

Yalow, R.S. & Berson, S.A. (1959). Assay of plasma insulin in human subjects by immunological methods. *Nature*, Vol. 184, No. 4699, (November 1959), pp. 1648-1649, ISSN 0028-0836

Yalow, R.S. & Berson, S.A. (1960). Immunoassay of endogenous plasma insulin in man. *The Journal of Clinical Investigation*, Vol. 39, No. 7, (July 1960), pp. 1157-1175, ISSN 0021-9738

Zhang, X.; Mobley, N.; Zhang, J.; Zheng, X.; Lu, L.; Ragin, O. & Smith, C.J. (2010). Analysis of agricultural residues on tea using d-SPE sample preparation with GC-NCI-MS and UHPLC-MS/MS. *Journal of Agricultural and Food Chemistry*, Vol. 58, No. 22, (November 2010), pp. 11553-11560, ISSN 0021-8561

Zhou, Q.; Ding, Y. & Xiao, J. (2006). Sensitive determination of thiamethoxam, imidacloprid and acetamiprid in environmental water samples with solid-phase extraction packed with multiwalled carbon nanotubes prior to high-performance liquid chromatography. *Analytical and Bioanalytical Chemistry*, Vol. 385, No. 8, (August 2006), pp. 1520-1525, ISSN 1618-2642

23

IPM Program to Control Coffee Berry Borer *Hypothenemus hampei*, with Emphasis on Highly Pathogenic Mixed Strains of *Beauveria bassiana*, to Overcome Insecticide Resistance in Colombia

Pablo Benavides, Carmenza Góngora and Alex Bustillo
Centro Nacional de Investigaciones de Café - Cenicafé
Colombia

1. Introduction

Coffee is cultivated by some 20 millions farmers in more than 50 countries in Africa, Asia and America, and generates an industry that surpasses USD 70 billion annually. Coffee cultivars are planted in a very wide range of ecological and social conditions and may be grown using few culture practices where almost no changes occur to the natural environment or by means of mechanization, irrigation, fertilization and pest control with chemical insecticides which often leads to a complete destruction of the surrounding vegetation and changes in the ecosystem. Small farmers are the predominant coffee growers in all countries and technology in most cases is not readily available to them. This coffee world situation results in a great variety of sanitary problems, which varies according to the country even in similar ecological regions. All agricultural production systems have to deal with plant protection problems and coffee is no exception. Since the early 20th century, coffee production suffers from numerous insect pests, being the coffee berry borer, the white stem borer, leaf miners and mealybugs, the most serious examples since they can cause coffee farmers to lose up to 20% of a crop and reduce the coffee value by 30 to 40%. These coffee pests could be kept below economic threshold levels by adopting integrated management strategies such as anticipation and continuous monitoring of pest outbreaks, maintenance of optimum shade, pruning of coffee bushes, good harvesting and processing of the berries, conservation and augmentation of indigenous natural enemies, introduction of exotic natural enemies and timely use of need based chemical or bio insecticides. This chapter deals with the most important insect pest affecting coffee plantations in all coffee producing countries, the Coffee Berry Borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae: Scolytinae), a brief description of an integrated pest management program that can help reducing the insect populations, the appearance of resistant insects in the field after spraying inadequately endosulfan for several years and a description of a novel strategy to select a highly pathogenic mixed strains of fungi in order to overcome this resistance and maintain infestation in the field under threshold levels.

2. Life cycle and damage caused by *Hypothenemus hampei*

The life cycle of Coffee Berry Borer has been studied by several authors (Corbett 1933; Bergamin 1943; Ticheler 1963; Baker 1984; Muñoz 1989; Decazy 1990a; Baker *et al.* 1992; Jaramillo *et al.* 2009), under different temperature and ambient conditions. In Colombia several studies have been carried out under laboratory and field conditions (Montoya y Cárdenas 1994; Gaviria *et al.* 1995; Ruíz *et al.* 1996; Ruíz 1996). The adult female coffee berry borer is a small black beetle, 1.5 mm in length, longer and slightly wider than the male. Its entire immature life stages are spent inside the coffee berry. Males mate inside the berry with females and never emerge. Males live between 50 and 75 days, while females from 100 to 150 days. The female beetle enters the coffee berry and borers a tunnel inside the coffee bean where lays eggs at a rate of 2-3 per day up to 20 days. The average number of offspring produced per female is 74 and the total life cycle was calculated to be 27.5 days (24.5°C), however, a complete development of coffee berry borer from egg to adult in Colombia is estimated between 45 to 60 days under conditions of 21ºC and 19ºC, respectively (Ruiz 1996, Bustillo 2002). The life cycle in degree–days is 237.2 with a threshold temperature development of 16.5 ºC. Even though there are reports of non-mated females giving origin to fertile eggs (Montoya y Cárdenas 1994, Muñoz 1989, Barrera *et al.* 1995), this has not being verified experimentally (Alvarez y Cortina 2004). Mated females emerge to fly and look for a new berry. Host finding is achieved initially by responding to volatiles emitted by the coffee berry during its development and close orientation to the berries may be assisted by vision preferring the red berries (Bustillo *et al.* 1998).

Hypothenemus hampei has a reproductive behavior that ensures a high degree of inbreeding. Female-biased sexual ratio was estimated to be 1:10 males to females (Bergamin 1943). Females mate with their flightless male siblings when still inside the berry, so they leave the infested berry already fertilized. Although cytological examination of somatic cells in males proved that they were diploid, males failed to express paternally derived alleles and then transmitted only their maternally derived chromosomes; thus, *H. hampei* is functionally haplo-diploid (Brun *et al.* 1995). It has been suggested that the unusual sex determination and skewed sex ratios favoring females is caused by the infection of *Wolbachia* in *H. hampei* (Vega *et al.* 2002, Benavides 2005); However, the presence of males in the coffee berry borer populations can be explained by the presence of an extra chromosome in male cells (Bergamin and Kerr 1951).

The damage caused by *H. hampei* is mainly a decrease of coffee yield due to abscission of berries, loss of weight, and a decrease on coffee quality and, therefore, coffee price. It has been estimated that there is a weight loss of 55% on coffee grains attacked by *H. hampei* (Montoya 1999); however, the decrease of weight of the total coffee production is about 18% Borbón 1990). *Hypothenemus hampei* also attacks young berries in formation (less than 20 weeks after flowering) which results in the abscission of 32% of coffee berries (Mendoza 1996). Furthermore, *H. hampei* causes yield losses as high as 40-80% at a field infestation of 90% (Le Pelley 1968). Coffee prices are greatly reduced when the beans exhibit *H. hampei* damage. International marketing policies do not allow coffee for exportation that have more than 1.5% damage caused by insects. Thus, the price of coffee in producing countries is severely reduced if the levels of infestation with *H. hampei* are greater (Duque and Baker 2003).

3. Distribution and dispersion of *Hypothenemus hampei*

The coffee berry borer dispersal through the world has been documented (Benavides 2005; Benavides *et al.* 2005), using molecular tools based on AFLP. Results suggested that invasion in Asia and America came from West Africa. The distribution of the fingerprints and its genetic relationship determined by a neighbor - joining analysis, showed that there were two introductions of Coffee Berry Borer in Brazil, then dispersed into American countries and a third introduction was evident in Peru and Colombia (Benavides 2005).
Hypothenemus hampei has now invaded all coffee producing countries worldwide (Table 1). It was first detected in Colombia in 1988 and is found now in all Colombian coffee plantations infesting near 800.000 ha and affecting the assets of more than a half million of Colombian coffee producing families. The coffee cultivars in Colombia have been kept free of important insect pests since the beginning of its development as a commercial exploitation. Only a few records exists on the attack of minor pests such as: *Leucoptera coffeellum* (Guerin - Méneville), *Coccus viridis* (Green), *Planococcus citri* (Risso), *Dysmicoccus brevipes* (Cockerell), *Puto barberi* (Murillo), and the red spider mite *Oligonychus yothersi* McGregor (ICA 1989; Cárdenas 1983, 1985). These arthropods have not became serious pests due to the fact that these agroecosystems are quite stable with a great biodiversity, which favors the development of beneficial agents and maintain in equilibrium the potential pests present in the farms. On the other hand, in the coffee growing areas, insecticides were not used indiscriminately and it is recognized that Colombia is the only country in the world where the coffee plantations were handled without the use or with very low use of insecticides up to the arrival of *H. hampei* (Bustillo 1991). This situation of equilibrium has been affected with the presence of this pest.

4. Implementation of an Integrated Pest Management for the control of the Coffee Berry Borer in Colombia

Different strategies are needed to control the Coffee Berry Borer, such as: cultural practices, crop agronomical management, which can reduce insect populations, the protection of beneficial fauna, and the introduction of exotic natural enemies and entomopathogens. Among these are the parasitoids: *Cephalonomia stephanoderis* Betrem, *Prorops nasuta* Waterston, *Phymastichus coffea* La Salle and the fungus *Beauveria bassiana* (Báls.) Vuillemin (Bustillo 1991, 1995; Benavides *et al.* 1994; Orozco 1995; Orozco y Aristizábal 1996). These strategies are covered by the concept of Integrated Pest Management (IPM) (NCA 1969; Rabb y Guthrie 1970; Andrews and Quezada 1989). The IPM focuses in a series of principles and concepts on pest control which are integrated and in a theoretical way are proposed to establish an ecological guideline in the solution of a pest problem. So the IPM is flexible, dynamic and always susceptible of improvement, even though its comprehension and adoption by the farmers may be difficult. In the case of *H. hampei* the IPM program has been defined as: the use of a series of control measures (cultural, biological and chemical) to reduce coffee berry borer populations to levels which can not cause economical damage and which allows the farmers the production of coffee for exportation in a competitive way. The control measures used must be compatible and should not cause deleterious effects to the farmers living in the coffee zone, nor to the fauna, and do not contaminate the coffee ecosystem. (Bustillo *et al.* 1998).This concept is now extended to the Integrated Crop Management

(ICM), which includes besides all the above mentioned tools, all the agronomical practices which are not directly pointed to the borer control, but if they are implemented can contribute indirectly to reduce borer populations (Bustillo 2002).

The implementation of an IPM program for the control of the Coffee Berry Borer in Colombia begins with sampling and determining an economic threshold level. The damage caused by Coffee Berry Borer creates the necessity to take efficient control measures, in the right moment when the insect menaces the coffee crop. Therefore, an important requirement in an IPM program is to measure in the field the insect population and correlate this population with the final damage to the crop. In the case of *H. hampei,* the sampling consists on taking, from a hectare (sampling universe), 30 trees randomly (sample size), selecting a productive branch containing 30 to 100 coffee berries (sampling unit) and then counting total number of berries in the branch and total number of berries infested by the Coffee Berry Borer. The infestation level is the result of dividing the total number of infested berries over the total counted coffee berries (Bustillo *et al.* 1998, Decazy 1990b, 1990c, Baker 1989, Baker *et al.* 1989, Muñoz 1988). The sampling should be done in a monthly basis in each coffee plot to follow up the borer populations and deciding control measures timely (Bustillo *et al.* 1998). By going through the coffee plots, allows to the evaluator localize sites where there is a high concentration of insect borer population, and in these marked places the farmer should intensify the control measures. On the other hand, when evaluation takes place, a sample of 2 – 3 infested berries per tree should be taken, to determine the borer internal population and mortality, recording also the position, inside or outside the coffee berry (Bustillo *et al.* 1998). The level of infestation, the position of the borer inside the berry, and the location inside the plot, allows the farmer to take good insect control decisions (Bustillo *et al.* 1998, Bustillo 2002). The infestation levels cannot surpass 2% in field conditions during the critical period which is described as the moment when the coffee berries are most susceptible to the insects attack such as 120 days after flowering.

The basis of the IPM to control *H. hampei* is Cultural Control (Benavides *et al.* 2002). It has been demonstrated that in coffee plantations after harvest, 10% of the coffee berry production remain on the trees and in the ground (Chamorro *et al.* 1995). If this population of berries is infested with the Coffee Berry Borer, then the insect can continue its reproduction. Cultural control consists then on timely harvesting the coffee berries before they drop onto the ground. If needed, coffee berries should be hand picked from the ground or using engine powered devices (Figure 1) (Bustillo 2002). The over mature berries and especially the dry ones, when infested by Coffee Berry Borer are the reservoir for borer populations that will infest the next coffee berry production. The dispersal of Coffee Berry Borer adults should be avoided (Benavides 2010), since 64 to 75% of the total population of Coffee Berry Borer individuals are taken to the processing area during harvest time (Moreno *et al.* 2001) and then fly back to the field (Castro *et al.* 1998). Studies in Colombia demonstrated that the timely harvest and collection of ripe berries left by the pickers, reduced levels of infestation from 70% to less than 6% during a coffee production cycle (Saldarriaga 1994, Peralta 1995). Later studies have shown that it is feasible to improve the efficacy of harvesters by allowing them to leave on the trees less than five ripe coffee berries, after a harvest pass (Díaz y Marín 1999). This has been also proved under a farmer's participatory research approach (Aristizábal *et al.* 2002, 2004a).

Region	Country	Year reported	Reference
Africa	Gabon	1901	(Beille 1925)
	Chad	1902	(Sponagel 1994)
	Dem. Rep. Congo	1903	(Leplae 1928)
	Central African Republic	1904	(Breilid et al. 1997)
	Uganda	1908	(Gowdey 1910)
		1922	(Beille 1925)
	Kenya	1928	(Abasa 1976)
Asia	Java	1909	(Hagedorn 1910)
	Sri Lanka	1910	(Vernalha et al. 1965)
	Sumatra	1919	(Corbett 1933)
	Malaysia	1929	(Corbett 1933)
	New Caledonia	1948	(Bugnicourt 1950)
	Philippines	1960	(Breilid et al. 1997)
	Fiji	1961	(Breilid et al. 1997)
	Tahiti	1963	(Johnston 1963)
	India	1990	(Sreedharan et al. 1994)
	Vietnam	1969	(Schmutterer, H. 1969)
America	Brazil	1922	(Bergamin 1946)
	Surinam	1960	(Bustillo et al. 1998)
	Peru	1962	(Amaral 1963)
	Guatemala	1971	(Hernandez and Sanchez 1978)
	Honduras	1977	(El Cafe de Nicaragua 1979)
	Bolivia	1978	(Romero 1990)
	Jamaica	1978	(Reid 1983)
	Mexico	1978	(El Cafe de Nicaragua 1978)
	Ecuador	1981	(Ruales 1997)
	El Salvador	1981	(Decazy 1987)
	Nicaragua	1988	(Monterrey 1991)
	Colombia	1988	(Bustillo 1990)
	Dominican Republic	1995	(Guharay and Monterrey 1997)
	Venezuela	1996	(Rosales et al. 1998)
	Costa Rica	2001	(Promecafe 2002)
	Puerto Rico	2007	http://uprm.edu
	United States	2010	http://hawaii.gov

Table 1. Worldwide distribution of *Hypothenemus hampei.*

Fig. 1. Vacuum machine prototype to collect coffee berries from the ground.

There are several tasks which need to be implemented in the farms to contribute to Coffee Berry Borer population reduction (Bustillo 2002): (1) planting resistant varieties such as Castillo®, which is resistant to coffee leaf rust (Alvarado and Moreno 1999), then does not require the use of fungicides and then allows the use of entompathogens to biologically control *H. hampei*, besides its coffee berries are more heavily attached to the tree and they do not fall as easily onto the ground; (2) planting the coffee trees in the field using larger distances among trees in array of 2 x 1 m and leaving two stems per tree (Mestre y Salazar 1995) allow workers to move around the coffee fields more efficiently to perform different tasks such as harvest, evaluation of infestation, sprays to control the borer, among others; (3) cutting down old trees in a planned coffee renovation is recommended after the fifth harvesting year (Mestre y Ospina 1994a, 1994b), this will ensure a proper picking procedure and would allow the implementation of cultural control practices; (4) The use of a weed selector (Figure 2) (Rivera 1997, 2000) in order to control unwanted weeds in the coffee plantations and leaving those that do not compete with the coffee plant, are good nectar producers and feed natural enemies of coffee insect-pests (Salazar and Baker 2002); and (5) avoiding the return to the coffee fields of flying Coffee Berry Borers by means of using traps (Aristizábal *et al.* 2002), depulping the coffee without water during the coffee processing (Alvarez 1991) and using devices to dry coffee beans using solar or mechanical energy (Benavides 2010b).

Besides Cultural control and agronomical practices, there is need to complement the IPM program with biological natural agents to control the Coffee Berry Borer, as well as the use of chemical insecticides in a safe and timely manner. Thus, Biological control plays an important role in this program, by means of using native beneficial fauna, exotic imported parasitoids and entomopathogenic fungi and nematodes. Native predators, parasitoids, nematodes and entomopathogenic fungi have been described in Colombia (Bustillo 1995, Vera *et al.* 2007, Lara *et al.* 2004). These findings confirmed the importance of preserving the Colombian coffee ecosystem with control measures that would not affect the beneficial fauna, so the farmers have to spend less effort in the control of this insect. Ants are playing an important role in the biological control of *H. hampei*. Vélez (2002) found *Solenopsis geminata*, *Dorymyrmex* sp., *Pheidole* sp., *Mycocepurus smithii* and *Gnamptogenys* sp. (Figure 3)

Fig. 2. Weed selector to maintain green covertures on the coffee soil to avoid erosion and favor biodiversity of beneficial fauna.

being capable of preying borer adults while attacking coffee berries. Armbrecht and Gallego (2007) also experimentally tested high predation levels of ants on *H. hampei* inhabiting berries on the soil.

Fig. 3. Ant of the genus *Gnamptogenys* preying on coffee berry borer adults boring into coffee berries (Photo G. Hoyos).

Other Biological control strategy is the introduction of natural enemies not present in Colombia. Three parasitoid species were introduced from Africa via quarantine in England:

Cephalonomia stephanoderis Betrem, *Prorops nasuta* Waterston y *Phymastichus coffea* La Salle. Massive production systems of these species have been documented (Orozco 1995, Portilla y Bustillo 1995, Orozco y Aristizábal 1996, Bustillo *et al.* 1996, Orozco 2002). The methodologies of these processes were made available to 11 private laboratories and the production of about 2000 millions was contracted by the Coffee Growers Federation during a period of five years (1995 – 1999). About 1700 millions of these parasitoid species were released in coffee infested fields throughout the country, with the initial purpose of establishing them (Bustillo *et al.* 1998). Field studies have shown the potential of *C. stephanoderis* and *P. nasuta* to reduce infestation levels of Coffee Berry Borer (Salazar y Baker 2002; Bacca 1999; Benavides *et al.* 1994; Aristizábal *et al.* 1997). A similar program was conducted with *Phymastichus coffea,* adult parasitoid of *H. hampei.* A massive production system was developed (Orozco and Aristizábal 1996, Orozco 2002) and after testing its selectivity to other Scolytinae species (López – Vaamonde *et al.* 1997), field releases were authorized in Colombian coffee plantations. *P. coffea* parasitize the *H. hampei* adult that is entering the coffee berry (Figure 4), being an ideal complement to the other two species. Under field conditions this parasitoid has a high searching capacity for *H. hampei* populations (Vergara *et al.* 2001a, Echeverry 1999), even at low population levels (< 5% infestation) (Vergara *et al.* 2001b); and greater parasitism when the borer is penetrating berries of 70 to 170 days of development (Jaramillo *et al.* 2002, 2005). Aristizábal *et al.* (2004b) showed the importance of these parasitoids in the regulation of Coffee Berry Borer populations. However, samples taken from releasing sites three years later did not confirm establishment of this species. Recent studies have shown only the field recovery of *P. nasuta*, in coffee plantations in Colombia (Maldonado and Benavides 2007). Apparently, this species is best adapted to conditions in the Neotropic. Even though mass produce the Coffee Berry Borer parasitoids is expensive, current efforts are made to rearing *H. hampei* on artificial diets (Ruíz *et al.* 1996, Portilla and Bustillo 1995, Portilla and Streett, 2006) then producing the parasitoids in a more cost effective procedure.

Fig. 4. *Phymastichus coffea* adult parasitizing an adult of *H. hampei* entering the coffee berry (Photo G. Hoyos).

Also, insect nematodes are considered a good alternative to decrease the population of Coffee Berry Borer that is present in infested berries onto the ground. In Colombia, it has not

been recorded any natural infection by entomonematodes (Bustillo *et al.* 2002). However, the literature indicates records of *Panagrolaimus sp*. (Panagrolaimidae) and *Metaparasitylenchus hypothenemi* Poinar (Allantonematidae) nematodes infecting borer populations naturally in coffee plantations in India and Mexico (Varaprasad *et al.* 1994, Castillo *et al.* 2002). Research on the nematodes *Steinernema colombiense* López and *Heterorhabditis bacteriophora* Poinar, found in the soil of Colombian coffee ecosystems (López *et al.* 2008) have focused to determine the pathogenicity on the Coffee Berry Borer, its behavior and strategy of host finding (Molina and López 2002; 2003), life cycle (López 2002), evaluation of application systems (Lara and López 2005) and evaluations under greenhouse and field conditions in small scale (Giraldo, 2003; Lara *et al,* 2004). Other studies cover evolutive relationship and species diversity of nematodes in Colombia (López *et al.* 2007). The species *S. colombiense* and *H. bacteriophora* are able to find and infect coffee berries infested by *H. hampei* (Lara *et al.* 2004). In Colombia, in spite of development of massive techniques in some countries as Germany and United States to produce entomonematodes, they are only produced in small scale using live insects such as *Galleria mellonella*, which made the process too expensive. This is a limitation if the market demands these organisms.

The cosmopolitan entomopathogenic fungus *Beauveria bassiana* is found naturally infecting the Coffee Berry Borer (Posada and Bustillo 1994). It has been sprayed in field conditions after artisan (Antía *et al.* 1992, Marín and Bustillo 2002) and industrial production (Morales *et al.* 1991). The Coffee Growers Federation in Colombia financed a large program to disseminate *B. bassiana* in all regions where the insect was dispersing (Bustillo 2002). The development of bioassays (González *et al.* 1993, Posada *et al.* 2002) to select virulent isolates, the instructions to reactivate the fungus in insects (Bustillo and Marín 2002) and the protocols for quality control of fungus produced using artisan and industrial processes (Vélez *et al.* 1997), have allowed a better control and improvement on the commercially available fungi formulations. Several studies on the efficacy of *B. bassiana* under field coffee conditions have been carried out (Bustillo *et al.* 1991, 1995, Bustillo and Posada 1996, Flórez *et al.* 1997). Results are variable and influenced by the quality and concentration of the fungus, climatic and crop management conditions. Levels of control may fluctuate from very low values near to 20% to high levels of 75%.

Current researches are now directed to improve the efficacy of these fungi to control the Coffee Berry Borer. Studies have been conducted on selection, characterization of isolates of *B. bassiana* and *M. anisopliae*, having in consideration their morphology (Padilla *et al.* 2000), pathogenicity (Jiménez 1992, Bernal *et al.* 1994), physiological characteristics and reproduction (Valdés *et al.* 1999, Vélez *et al.* 1999, 2001) and using molecular techniques (Valderrama *et al.* 2000, Gaitán *et al.* 2002). Recently, there is interest in the genetic transformation of these fungi (Góngora *et al.* 2000, Góngora 2005, Rodríguez and Góngora 2005) and also *Metarhizium anisopliae* (Pava *et al.* 2008), with genes that could increase its virulence and be more efficient under field conditions to control the Coffee Berry Borer (Góngora *et al.* 2000, Góngora 2005, Rodríguez and Góngora 2005), but there are not yet regulations in Colombia to manipulate transgenic microorganisms, which delays the advances in this area. On the other hand there are evidences of more efficient entomopathogens under field conditions by spraying mixtures of different strains to control *H. hampei* (Cárdenas *et al.* 2007).

The use of insecticides to control the Coffee Berry Borer should be carried out only when technically needed, that is when levels of borer infestation surpasses 2% during the critical period of the attack of the Coffee Berry Borer, and at the moment that more than 50% of the

flying females are still outside the coffee berries. These two parameters are obtained with the proposed sampling above mentioned (Bustillo 2002). After testing more than 50 chemical insecticides, three less toxic (category III) molecules were recommended for achieving similar efficacies than endosulfan: fenitrotion, phenthoate and chlorpyrifos (Villalba *et al.* 1995). They should be applied in a localized area where the insect is present and using the appropriate spray technology to achieve a good borer control (Villalba *et al.* 1995, Bustillo *et al.* 1998, Posada *et al.* 2004). In field conditions the control of *H. hampei* with chemical insecticides is very erratic. To explain failures different factors need to be taken in consideration such as correct dosage, calibration of equipment and operators, field topography, environmental conditions at the moment of spraying, and the proper moment to apply the insecticide according to the borer attack.

Impact of our research on *H. hampei* in the Colombian coffee industry is supported by the statistics of Almacafé, the coffee organization in charge of coffee storage and exportation (Figure 5). Levels of infested green coffee by this insect in Almacafé have been reduced greatly. In 1994 infestation average levels were about 16% of all stored green coffee, in 2002 were below 4.1% (Abisambra 2004) and in 2007 dropped to 2.1% (FNC 2007). The adoption of coffee management strategies to reduce populations of *H. hampei*, have contributed to the commercialization of the Colombian coffee without any obstacle and favored the coffee economy which at current prices could represent savings around US$120 million dollars annually. The social impact can be summarized as the preservation of the environment for using less toxic insecticides and no toxic bioinsecticides, reducing costs and maintaining high coffee quality in the market.

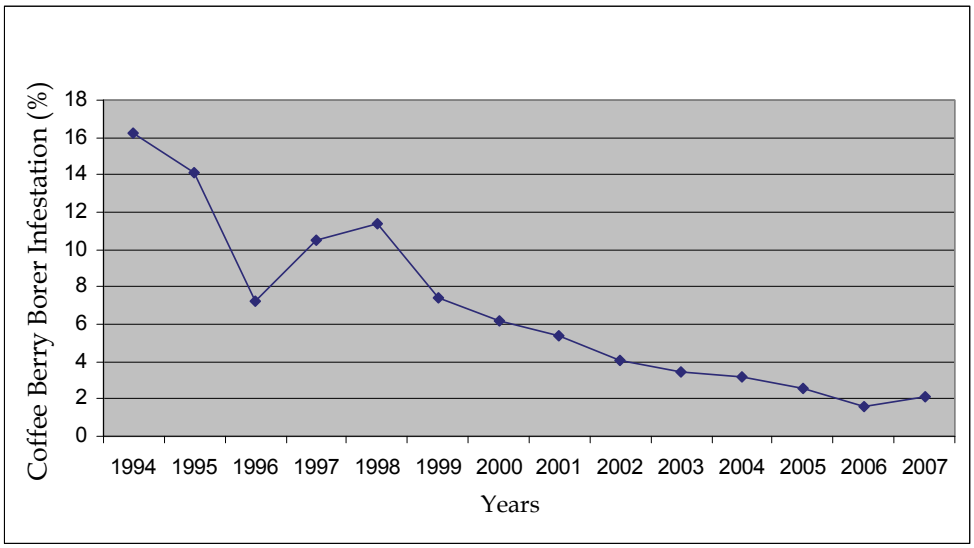


Fig. 5. Records of percentage of dry parchment coffee infested with *H. hampei*, stored in Almacafé, Colombia from 1994 o 2007. (Reports of Almacafé).

Unfortunately, irrational use of insecticides has caused several problems, such as insect resistance (Brun *et al.* 1989). This situation is accentuated by the sib-mating behavior and the functional haplodiploidy exposed by the Coffee Berry Borer (Benavides *et al.* 2005), which allows the fixation of mutations in few generations of this matrilineal species. This is the case of resistant of *H. hampei* to endosulfan reported initially in New Caledonia (Brun *et al.* 1989), tested later through molecular studies (Ffrench-Constant *et al.* 1994) and found that depends on the gene *Rdl* which codifies a subunit of the receptor of acid γ-aminobutiric (neurotransmitter GABA), that is responsible to activate the chloro channel during synapses. This gene of resistance was favored in New Caledonia through selection processes. Insecticides belonging to the group of cyclodienes as DDT, lindane and endosulfan, were continuously applied in a generalized manner since 1966 and in less than 20 years, levels of Coffee Berry Borer infestation reached the historic maximum and the resistance was documented (Brun *et al.* 1989). This resistance has also been described in Colombia (Góngora *et al.* 2001, Navarro *et al.* 2010). The appearance of this mutation in *H. hampei* population in Colombia followed not appropriate control practices performed by few non-adopting IPM coffee farmers that sprayed irrationally endosulfan for several years, thus the frequency of this gene in the borer population increased and chemical insecticide to control Coffee Berry Borer failed. Initially, in order to confirm the presence of the Dieldrin resistance allele (Rdl^R) gene in Colombian Coffee Berry Borer populations, concentration-mortality responses for individual Coffee Berry Borer lines reared from four different Colombian coffee areas were estimated using a Potter Spray Tower (Burkard Manufacturing Co). Three different concentrations of endosulfan were tested on insects reared from coffee areas where endosulfan resistant was suspected: low dosage of 400 ppm, medium dosage of 10,000 ppm and high dosage of 20,000 ppm. Susceptible Putative (SS) insects were discriminated successfully from heterozygous putative (RS) and homozigous (RR) insects with the low and high dose. The low dosage caused a 100% mortality rate in the (SS) susceptible strains after 24 hours endosulfan exposure; all (RS) and (RR) survived. The survivors were then sprayed with either 10,000 or 20,000 ppm and evaluated after 6hrs. The 20,000 ppm dosage caused 79.3, 74.02, and 94.64% mortality in the (RS) strains from three studied sites. All homocigotes (RR) lines used as control survived the high dosage of 20,000 ppm. The individuals were then genotyped and the genetic condition was corroborated by PASA. The results obtained confirmed the presence of the gen *Rdl* in Colombian populations of *H. hampei.* PASA showed to be an appropriate technique to identify resistant populations from the field, based on this, a melting temperature (T_m) shift genotyping method that relies on allele-specific PCR was described for insecticide resistance-associated single nucleotide polymorphism (SNP) at the *H. hampei Rdl* gene (Navarro *et al.* 2010). Later findings on the resistant populations have shown biological disadvantages associated to the borer individuals. Homozygous resistant lines showed a marked decrease on progeny production while compared to homozygous susceptible individuals, as well as a longer survivorship time of the resistant borers (Figure 6 and 7).

Right now it is considered that in view of the market restrictions on insecticide residues in exporting commodities and with the emphasis on specialty coffees, environmentally friendly strategies such as *B. bassiana* are now considered as a very valuable alternative in the reduction of *H. hampei* (Bustillo 2004).

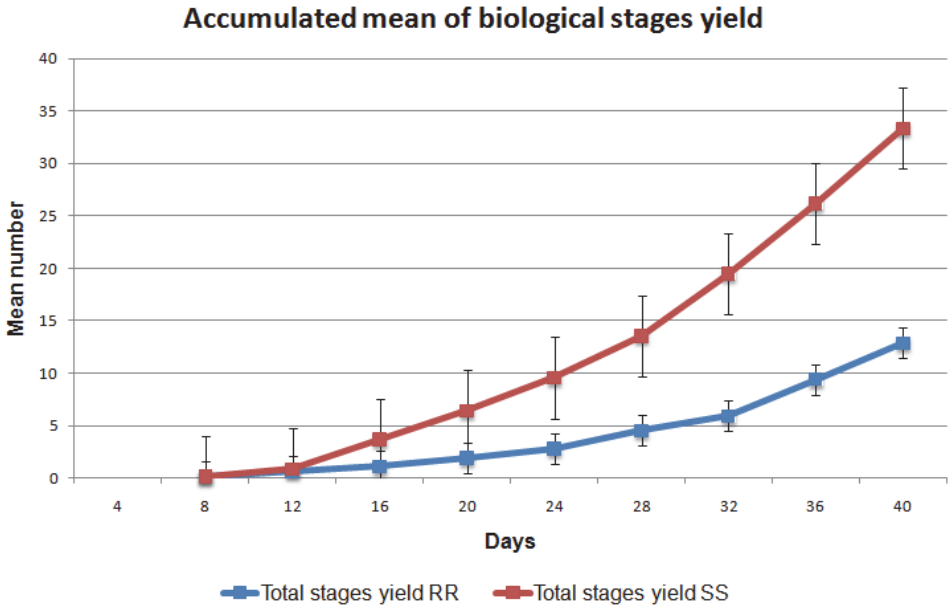


Fig. 6. Total progeny produced by Coffee Berry homozygous resistant RR and susceptible SS to endosulfan insecticide in lab conditions.

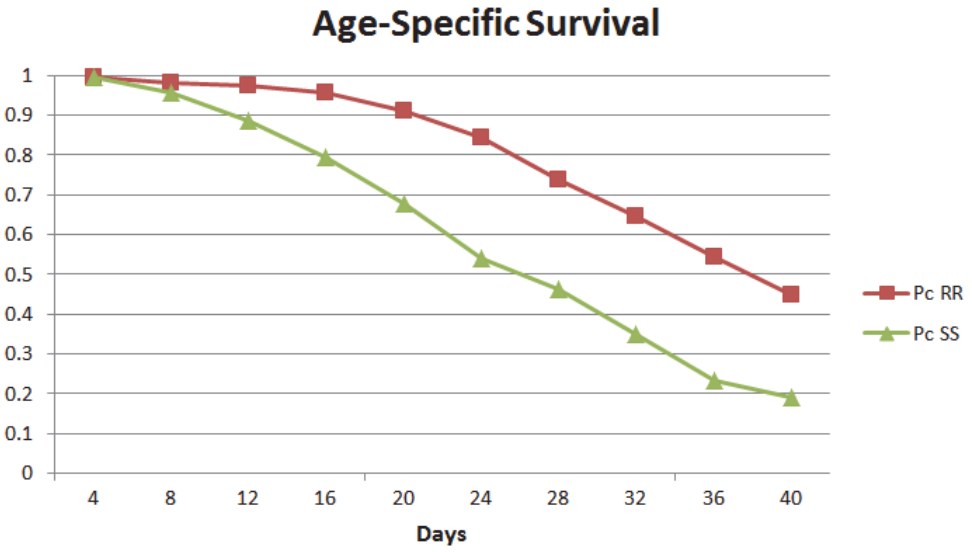


Fig. 7. Survivorship of adult Coffee Berry homozygous resistant Pc RR and susceptible Pc SS to endosulfan insecticide in lab conditions.

5. *Beauveria bassiana* as a strategy to overcome insecticide resistance

The emergence of chemically resistant insects as well as the desire to decrease reliance upon chemical pesticides in favor of more eco-friendly and organic production compatible methods has lead in Colombia to the investigation of alternate biologically based control strategies, one of which is the use of entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillemin, which is actively being examined as a biological agent to control the Coffee Berry Borer.

Indeed, in the Colombian coffee ecosystem, this fungus is a natural controller of the *H. hampei* (Bustillo 2006). Furthermore, there are no known diseases of *H. hampei* caused by either bacteria or viruses. High Coffee Berry Borer mortality caused by the fungus *B. bassiana* has been reported on Indian coffee plantations (Haraprasad et al 2001) and Mexico (De La Rosa et al 2000) as well.

B. bassiana is a broad host range insect pathogen that has been EPA approved for use as an insect pest biological control agent and is available from various commercial companies world-wide (Goettel *et al.*, 2005) including Colombia.

Since the arrival of the Coffee Berry Borer to Colombia, *B. bassiana* has been related to the insect and it was reported attacking insects since 1990 (Velez and Benavides, 1990). Today, the fungus is considered a natural controller of the pest because it is found infecting the insect in all the countries where Coffee Berry Borer has arrived.

However in order to get to this levels, when the insect arrived, the research developed in Cenicafé, allowed to produce enough fungi, to spray the coffee-invaded area, free of cost to the farmers by the Coffee Growers Federation during three years. This program allowed the Coffee Berry Borer to be exposed to the fungus causing infections on its population. This is a good example of classical biological control through the introduction of a beneficial organism not present in the insect population. Between 1992 and 1995, 200 tons of fungi were produced ("Strain Cenicafé") by the Colombian Coffee Growers Federation and private laboratories. Between 1996 and 1998 this production was raised to 400 tons to control the borer. The developments of this research allowed the formation of several private laboratories in Colombia dedicated to the production of several species of entomopathogenic fungi not only to control coffee pests but several other insect pests of different crops. Biological commercial laboratories from Colombia have founded new laboratories in other Latin American countries such as: Ecuador, Brazil, Peru, Panama, Costa Rica and Guatemala.

Today the natural control caused by the fungus in the Colombian coffee zone has been calculated around 10%. If *B. bassiana* has not been present, the economic losses in the Colombian coffee industry will be much higher (Góngora *et al.*, 2009). Today *B. bassiana* is part of the Coffee Berry Borer - IPM strategy and its application is recommended by Cenicafe (Bustillo 2002).

However the use of high concentrations of *B. basassina* spores is costly and one of the ways of reducing the biological control cost is to increase the virulence of the strains and its resistance to adverse environmental conditions. This will allow the reduction of the spore doses required for controlling the insect and to diminish the mortality time, in such a way that the insect will cause a minor damage in the berries, helping to reduce the problem of the delaying in mortality caused by the fungus comparing to the chemical insecticides (St Leger and Wang 2010).

Historically fungal pathogens of plant and insect pests have not met expectations because of slow kill, failure to identify strains active at low doses and inconsistent results compared with the chemicals they compete with (Gressel 2007, Gressel *et al.*, 2007). These failures may be exasperated by our incomplete understanding of the biological and genetic factors that make fungi effective. However, lack of efficacy could also be inbuilt because an evolutionary balance may have developed between microorganisms and their hosts so that quick kill, even at high doses, is not adaptive for the pathogen (St. Leger and Wang, 2010).

The combination of geographical location and agricultural practices in their plantations throughout Colombia also, results in a wide variety of microclimate conditions that can affect the performance of a biological control agent (Cruz et al 2006). Coffee cultivation conditions in the country range in altitude from 1,000 to 1,800 m above sea level, with precipitations during the rainy seasons and solar intensities that change widely among locations and along the year (Cenicafe 2010). Similarly, farmers use plant densities that vary between 5,000 to 10,000 plants per hectare, with or without shade trees. This affects the consistency of biological control performance when compared to spraying synthetic insecticides under homogeneous cultivation conditions, decreasing the appeal for biological product applications by the farmer. Therefore, a constant improvement in biocontrol technology is required as the need to reduce economical and environmental costs of control measures plays a critical role in an agricultural industry such as coffee production.

As part of this goal "constant improvement in biocontrol technology" Cenicafé has collected throughout the years 196 isolates of *B. bassiana* from diverse hosts and geographic origins (Cruz et al 2006. Góngora et al 2009). Even though the worldwide population of this species has been found to have a low genetic diversity (St. Leger *et al.* 1992, Glare and Inwood 1998, Castrillo *et al.* 1998, Coates *et al.* 2002, Gaitan *et al.* 2002), *Beauveria*'s isolates show differences in their virulence (Bustillo and Posada 1996, Velez *et al.* 1999, Cruz 2006). Nevertheless, until now, only the isolate Bb 9205 has been the only genotype distributed for pest control purposes in Colombian coffee plantations.

We know that the current practice of production and application of clonal isolates selected because of their virulence towards an insect can result in a short and limited suppression of the pest (Boucias *et al.* 2000). Tigano-Milani (1995) proposed the hypothesis that more than one haplotype (clone) may be required to initiate and maintain an epizootic in a natural and heterogeneus insect population, such as the one found under Colombian conditions. The variability of the strains is the factor that would allow the fungus to adapt to changing environmental conditions and to successfully attack different insect populations.

Experimentally, however, the role of strain diversity may be hard to establish due to the difficulty to identify intraspecific variations using classical morphological and biochemical methods, and therefore making laborious the monitoring and tracking of multiple strains in the same infection. In this sense, DNA profiles have been used as powerful and sensitive tools to precisely identify individual strains infecting a host population (Wang *et al.* 2004), but to date only two assays using molecular markers on strain mixtures or coformulated strains of entomopatogenic fungi have been reported. Leal-Bertioli *et al.* (2000) distinguished two co-formulated strains of *Metarhizium* infecting *Phaedon cochleariae*, while Wang *et al.* (2004) differentiated two strains of *Beauveria* infecting *Galleria mellonella*. In both *in vitro* assays, one strain dominated over the other, and parasexual recombination or heterokaryon formation was detected. In nature, however, the presence of diverse strains of *B. bassiana* in

samples collected from the same pest outbreak has been reported, suggesting that successful epizootic development requires the involvement of genetically distinct genotypes (Castrillo *et al.* 1998). Besides, under field conditions, monitoring of massive applications of two *B. bassiana* strains resulted in co-infection or genetic recombination of the isolates (Wang *et al.* 2004).

Based on those hypothesis we selected ten *B. bassiana* strains, previously characterized by RAPDs (Gaitan *et al.* 2002). They were reactivated from Cenicafe's collection of entomopathogens. Six of these isolates came from various places in Colombia (Bb 9001, Bb 9005, Bb 9010, Bb 9011, Bb 9119, Bb 9205), and the rest came from Thailand (Bb 9016), Philippines (Bb 9020 and Bb 9023) and Canada (Bb 9024). Genomic DNA from the strains was characterized using ITSs and β-tubulin sequences as well as AFLPs markers (Cruz *et al.* 2006).

For the ITS: A PCR fragment containing the 3´end of the 18S ribosomal RNA gene, the complete ITS1, 5.8S and ITS2, and the 5´end of the 26S, was amplified using the primers ITS 1 and ITS 4 described by White *et al.* (1990. An ITS amplification product of around 569pb was obtained for all the isolates. The ITS sequences for each on of strains were deposited in the GenBank. For the β-tubulin sequences, a PCR reactions with the primers Bt-T2m-Up and Bt-LEV-Lo1, described by de Jong *et al.* (2001), were used to amplify the 3' end intron of the β-tubulin gene of *Beauveria* sp. and part of its flanking exons. All the isolates displayed a 982bp PCR amplification product, corresponding to the 3' intron and parts of the flanking exons.

A high number of informative and reliable sets of AFLPs for each one of the isolates was obtained. Only consistent bands with molecular weight between 200-500bp were scored to generate a binary matrix with 120 markers, and a PCOORDA analysis was done.

Based on the grouping analysis obtained with ITSs and β-tubulin, the isolates were clustered in three genetic groups. Group 1 made up by isolates: Bb 9001, Bb 9005, Bb 9010 and Bb 9020; Group 2 formed by isolates: Bb 9011, Bb 9016 Bb 9119 and Bb 9205, and Group 3 composed by isolates: Bb 9023 and Bb 9024. The cluster analysis also confirmed the low but significant intraspecific genetic diversity present among the strains.

Single strain virulence towards the Coffee Berry Borer under laboratory conditions were done, virulence tests were carried out according to the method described by Gonzalez *et al.* (1993). *H. hampei* adults were obtained from a laboratory colony maintained in parchment coffee (Bustillo *et al.* 1998). For each treatment, insects (15 individuals per treatment with 4 replicates) were inoculated by dipping them into a 10 ml spore suspension of $1x10^6$ spores ml^{-1}. They were then transferred to individual containers with a filter paper at 25 °C and 80% humidity. Insect mortality was recorded at 24 hours intervals during 8 days, discriminating between death by fungal infection, with the observation of mycelium on the cadaver, and death by other causes. The virulence ranged between 90% and 57.5%.

All the inoculations with mixtures resulted in coinfection events. Combinations of genetically similar strains showed no significant differences when their virulences were compared. However, mixtures of genetically different strains led to both antagonism and synergism. The lowest virulence percentage (57%) was obtained by putting together the most virulent strain of each group (Bb 9020, Bb 9023, Bb 9205), contrary to the highest virulence percentage (93%) that resulted from **mixing the three least virulent strains (**Bb 9001, Bb 9119, Bb 9024).

Based on those first results Cardenas *et al*., 2007, evaluated again the virulence of all the individual strains and mixtures, the virulence assay results under lab conditions are showed in Table 2.

Treatments	***H. hampei* Mortality caused by B. bassiana**	
	Average(%)	**C. V.(%)**
Bb 9020	81.67 bcd	4.99
Bb 9023	83.33 bc	6.19
Bb 9205	88.33 b	4.62
Bb 9001	76.67 cd	6.73
Bb 9119	73.33 de	7.04
Bb 9024	53.33 f	9.68
Mixture A- **most virulent strain** (Bb 9020, Bb 9023, Bb 9205)	65.00 e	8.43
Mixture B- **least virulent strains** (Bb 9001, Bb 9119, Bb 9024)	100.00 a	0.00
Commercial formulation	83.33 bc	6.19

Averages with no common letters indicate differences among treatments according to Tukey (P=0.05) comparison test.
C.V. Coefficient of variation

Table 2. Coffee Berry Borer mortality percentage caused by *B. bassiana* (1x10 ^{6} spores /ml) in lab conditions.

With the same strains and mixtures we evaluated the virulence under field conditions. For this, in the coffee farm "Tamboral", located in Manizales - Caldas, in a 20 month coffee plot Colombia variety, twenty-five-tree plots with three repetitions distributed through a completely randomized design were used. One coffee tree per plot and a branch with 50 coffee berries were selected to make artificial infestations with the insect. After 24h, the infested branches were sprayed using a dose of 2x10^{7} spores/branch for each treatment. After 30 days, the insects mortality was assessed through berries dissection.
In the coffee plantation, the highest mortality was registered with the low-virulence strain mixture (66.6%) witch it was higher than the mortality caused by individual strains or other mixture evaluated. So far a mortality of 66.6% is the highest observed due to *Beauveria* sp. under field conditions in Colombia (Table 3).
The results indicated the promising potential of designing strain mixtures as an alternative for the biocontrol of *H. hampei* and other pests, and provides tools for the understanding of the ecological dynamics of entomopathogen populations under natural conditions.
In addition, the problem of the Coffee Berry Borer is not only limited to the insect population that attacks the coffee berries from the tree branches but also exist a permanent insect population that remain in the berries that have fallen onto the ground, which are at the base of the trees after coffee harvesting and act as a driving source for new infestations (Castaño *et al.* 2005, Bustillo 2002, Benavides 2010a). The fallen berries are reservoirs for adult insects and are food for larvae. When conditions are appropriate, i.e. under high humidity and temperature conditions, adult insects that remain in the fallen berries fly to new coffee berries that are on the branches of the trees or that have fallen to the soil,

Treatments	Mortality(%)	
	Average	C. V.(%)
Bb 9020	53.1 b	8.7
Bb 9023	55.5 ab	16.2
Bb 9205	59.6 ab	11.4
Bb 9001	54.1 ab	15.5
Bb 9119	58.3 ab	16.4
Bb 9024	55.1 ab	21.9
Mixture A- **most virulent strain** (Bb 9020, Bb 9023, Bb 9205)	60.2 ab	11.5
Mixture B- **least virulent strains** (Bb 9001, Bb 9119, Bb 9024)	66.6 a	15.8
Commercial formulation	56.6 ab	17.7
Testigo dentro de la parcela	19.5	63.9
Testigo fuera de la parcela	8.4	63.7

Averages with no common letters indicate differences among treatments according to Tukey (P=0.05) comparison test.
C.V. Coefficient of variation

Table 3. Coffee Berry Borer mortality percentage caused by *B. bassiana* (1x10 ^{6} spores /branch) in field conditions.

penetrating growing or ripe berries and depositing eggs. The eggs hatch, and the larvae consume the seeds, damaging them and causing these berries to fall to the base of the tree. The larvae become adults, and those adults mate with the siblings and fly, repeating the entire cycle. Therefore, determining the efficacy of *B. bassiana* on Coffee Berry Borer populations that emerge from fallen infested berries and infest the berries in the trees will contribute to improving pest control strategies and decrease the losses caused by this pest.
Based on this, we evaluated the effect of the application of the *B. bassiana* to infested berries from the soil and its effect on the percentage of new berry infestations from the trees. The experiment was done in 2 different Colombian coffee experimental stations during 2009: Paraguaicito-Quindío and Naranjal-Caldas. The research at Paraguaicito was conducted between February and March. Paraguaicito is located at altitude of 1210 m above sea level, has during those months on average 23°C, 77% RH, and sandy-loam soils. The coffee trees were 3 years old in second harvest, with average size of 1.7 m, and they were planted at a distance of 1.30 m × 1.30 m. Research at Naranjal was conducted between July and August. Naranjal is located at altitude of 1381 m, has during that period of the year on average 21.4 °C and 68% RH, and clay-loam soils. The coffee trees were planted at a distance of 2.0 m × 1.0 m, and they were 2 years old after stump in second harvest. At both locations, *Coffea arabica* variety Castillo trees was used and they had berries of 120 to 150 days old. The plots had a slope of no greater than 20%.
The treatments consisted of application of *B. bassiana* strain Bb9205, a mixture of three *B. bassiana* strains Bb9001, Bb9119, and Bb9024, a commercial formulation, and a control (water) to infested berries placed at the base of a coffee tree. The experimental plot in each location was formed by 9 coffee trees in square with a 3 × 3 array, in which the central tree was the sample unit. Each treatment was replicated 10 times and forty experimental plots were established.

In the sample unit trees, all infested coffee berries on the tree branches and the fallen berries from the bases were removed. Then, 50 artificially infested coffee berries were placed on the ground next to the base of each tree. The coffee berries were infested with 4 adult females, 30 days previously to the set up of the experiment. The treatments were sprayed over the berries left on the ground one day later, the trees, and their bases were completely covered with net entomological cages. The four treatments were assigned according to a completely randomized experimental design.

After 30 days of establishing the treatments, the total number of berries per tree, the total number of infested berries, and the percentage of infestation per tree were recorded. Then, 50 infested berries were randomly collected from all branches on each tree and were dissected in the laboratory to register the position of the insects in the berries and the degree of penetration inside the berry. Positions A and B referred to Coffee Berry Borer individuals initiating the attack of the fruit, whereas positions C and D indicated that the insects were inside the seed (Bustillo 2006). Number of live Coffee Berry Borers, dead Coffee Berry Borers without the presence of fungus, and dead Coffee Berry Borers with signs of fungus were recorded. The dead Coffee Berry Borers with no fungus signs were placed in a moist chamber to add them into the insects killed by the fungus, if stated.

The results showed reduction on infestation levels ranged from 15 to 55% at Naranjal in all treatments with respect to the control. In Paraguaicito, there were differences in percentage of infestation between the mixture and the control, and the reduction was 38%. At Narajal the infestation decreased by 50% (2.2 fold) with treatment with the mixture of Cenicafé strains compared to the control, whereas the decrease in the percentage of infestation at Paraguaicito with the same treatment was 30% (1.6 fold) compared to the control treatment. At both locations, treatment with the mixture of Cenicafé strains had a greater effect on Coffee Berry Borer that emerged from the berries left on the ground, which caused a significant decrease in the percentage of infestation of berries in the tree.

In the berries dissected from treated tree, insect mortality was about 40% at both locations compared to 15% in the control and it also decreased the insect population inside the newly infested berries on the trees by 55 to 75% (Table 4). In general, we can conclude that the application of the fungus on infested berries left on the soil can decrease the number of individuals of subsequent generations of Coffee Berry Borers (F1) by up to 55% at Paraguaicito. At Naranjal, the decrease in the number of eggs reached 90% after treatment, and the larvae decreased up to 87% compared to the control. Overall, the entire population was reduced at least 75% after fungal treatment, with the mixture of Cenicafé having the greatest effect. Previously, Aristizábal (2005) stated that treatment of berries on the soil with *B. bassiana* affected the Coffee Berry Borer in such a way that reduced the progeny of biological stages of Coffee Berry Borers produced by these infected insects in tree berries, but until now, the quantification of this reduction had not been examined.

It has been reported that insects infected with an entomopathogenic fungus may alter their behavior during mating and oviposition (Goettel *et al.* 2005) decreasing their progeny. In the case of Coffee Berry Borer, the infection can cause physiological damage to insects in such a way that they cannot mate inside berries, or eggs do not develop after mating. Fungal infection can also induce aberrant behavior that can decrease the fitness of the insect. These behaviors include male copulating more with infected females, or infected females not copulating, which are behaviors previously reported in other species of insects (Watson and Petersen 1993; Roy et al 2006). The effect of strain mixture have been evaluated recently for other authors, mixture *Pseudomonas fluorescens* strains and *Enterobacter cloacae* have been

used to boost biocontrol efficacy and consistency of potato maladies – dry rot, late blight, pink rot, and sprouting (Slininger et al 2010), In nature, the presence of diverse strains of *B. bassiana* in samples collected from the same pest outbreak has been observed, suggesting that successful epizootic development requires the involvement of genetically distinct genotypes (Castrillo *et al.* 1998). Besides, under field conditions, monitoring of massive applications of two *B. bassiana* strains resulted in co-infection or genetic recombination of the isolates (Wang *et al.* 2004).

	Station Paraguaicito–Quindio			**Station Naranjal-Caldas**		
Treatment	**Eggs**	**Pupae**	**Larvae**	**Eggs**	**Pupae**	**Larvae**
Brocaril®	31.8* ± 10.72	0.0	37.7 ± 13.99	16.1* ± 6.22	0.4 ± 0.9	23.6* ± 10.11
Bb9205	58.3 ± 22.37	0.0	63.7 ± 31.05	29.9* ± 18.23	0.0	35.8* ± 16.7
Mixture	39.9 ± 21.85	0.4 ± 0.9	30.1 ± 20.87	14.6* ± 5.1	0.0	19.1* ± 12.17
Control	69.6 ± 25.93	0.0	63.2 ± 39.12	139.2 ± 27.25	0.0	149.1 ± 19.17

* Average statistical differences compared with the control according to Dunnett's test (P = 0.05) per biological state.

Table 4. Mean numbers of biological stages of the Coffee Berry Borer present in berries collected from the field. Effect of *B. bassiana* application on Coffee Berry Borer emerging from berries left in the field and on the Coffee Berry Borer offspring.

We concluded that *B. bassiana* significantly decreased Coffee Berry Borer populations that emerged from fallen, infested, coffee berries and reduced future insect generations and the mixture was the most effective for decreasing the insect populations.

6. Conclusions

The Coffee Berry Borer is a serious insect pest of coffee crops worldwide. Historically, the strategies to overcome this pest have not been aligned with environmentally friendly schemes and problems such us resistant to chemical insecticides arrived soon as expected.
An Integrated Pest Management program in Colombia has proved to maintain the Coffee Berry Borer under the economic threshold, however more progress on less labor intensive strategies are needed.
Biological control agents have been introduced, conserved and augmented in order to naturally control Coffee Berry Borer. None, except the use of highly pathogenic fungi such as *Beauveria bassiana*, has proved to be economically and biologically effective to control Coffee Berry Borer in the field. Our results helped in the understanding of Insect – entomopathogen interaction and the development of a mixture of strains that could be very important for insect control not only in coffee but also in other crops.

7. References

ABASA, R. O. 1976. A review of the biological control of coffee insect pests in Kenya. Kenya Coffee 41: 327-333

ABISAMBRA, A. 2004. Informe Gerente General, ICA. Oficina Asesora de Comunicaciones del ICA, Comunicado, Bogotá, Colombia, febrero 23, 2004, 2 p.

ALVARADO, G.; MORENO G. 1999. ¿Cómo se distribuye anualmente la cosecha de las variedades Caturra y Colombia? Cenicafé, Avances técnicos No. 260, Chinchiná, Colombia, 4 p.

ALVAREZ, J. 1991. Despulpado de café sin agua. Cenicafé, Avances técnicos No. 164, Chinchiná, Colombia, 6 p

ALVAREZ, J. E.; CORTINA, H. A. 2004. ¿Presenta partenogénesis *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae: Scolytinae)? Fitotecnia Colombiana (Colombia), 4 (1): 107-111.

AMARAL, S.F. 1963. Do El gorgojo de la cereza del cafe "Broca" y su combate. Cafe Peruano (Peru) 1:8-20

ANDREWS, K. L.; QUEZADA, J. R. 1989. Manejo integrado de plagas insectiles en la agricultura: Estado actual y futuro. Escuela Agrícola Panamericana, El Zamorano, Honduras, 623 p.

ANTIA, O. P.; POSADA, F. J.; BUSTILLO, A. E.; GONZÁLEZ, M. T. 1992. Producción en finca del hongo *Beauveria bassiana* para el control de la broca del café. Cenicafé, Avances técnicos No. 182, Chinchiná, Colombia, 12 p.

ARISTIZÁBAL, L. F. 2005. Investigación participativa en el manejo integrado de la broca del café. *In*: Memorias XXXII Congreso Sociedad Colombiana de Entomología, Socolen, Ibagué 27 – 29 de julio 2005. p. 65 – 71.

ARISTIZÁBAL, L. F.; BAKER, P. S.; OROZCO, J.; CHAVES, B. 1997. Parasitismo de *Cephalonomia stephanoderis* Betrem sobre una población de *Hypothenemus hampei* (Ferrari) con niveles bajos de infestación en campo. Revista Colombiana de Entomología, 23 (3-4): 157-164.

ARISTIZABAL, L. F.; BUSTILLO, A. E.; JIMÉNEZ, M.; TRUJILLO, H. I. 2004a. V Encuentro de caficultores experimentadores. Manejo integrado de la broca del café a través de investigación participativa. Convenio Colciencias – FNC – Cenicafé. Fundación Manuel Mejía, Chinchiná, Colombia, septiembre 21 y 22 de 2004, 70 p.

ARISTIZÁBAL, L. F.; SALAZAR, H. M.; MEJÍA, C. G. 2002. Cambios en la adopción de los componentes del manejo integrado de la broca del café, *Hypothenemus hampei* (Coleoptera: Scolytidae) a través de metodologías participativas. Revista Colombiana de Entomología, 28 (2): 153 -160

ARISTIZÁBAL, L. F.; SALAZAR, H. M.; MEJÍA, C. G.; BUSTILLO, A. E. 2004b. Introducción y evaluación de *Phymastichus coffea* (Hymenoptera: Eulophidae) en fincas de pequeños caficultores a través de investigación participativa. Revista Colombiana de Entomología, 30 (2): 219- 224.

ARMBRECHT, I.; GALLEGO, M. C. 2007. Testing ant predation on the coffee berry borer in shaded and sun coffee plantations in Colombia. Entomologia Experimentalis et Applicata, 124 (3): 261–267.

BACCA, R. T. 1999. Efecto del parasitoide *Prorops nasuta* Waterston (Hymenoptera: Bethylidae) sobre poblaciones de broca del café *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae). Santafé de Bogotá, Universidad Nacional de Colombia, Facultad de Agronomía. (Tesis Maestría en Ciencias Agrarias). 186 p.

BAKER, P. S. 1984. Some aspects of the behavior of the coffee berry borer in relation to its control in Southern Mexico (Coleoptera: Scolytidae). Folia Entomológica Mexicana, 62: 9-24.

BAKER, P. S. 1989. A sampling plan for a control project against the coffee berry borer (*Hypothenemus hampei*) in Mexico. Tropical Pest Management, 35 (2):169-172.

BAKER, P. S.; BARRERA, J. F.; RIVAS, A. 1992. Life history studies of the coffee berry borer (*Hypothenemus hampei*, Scolytidae) on coffee trees in southern Mexico. Journal of Applied Ecology, 29: 656-662.

BAKER, P. S.; BARRERA, J. F.; VALENZUELA, J. E. 1989. The distribution of the coffee berry borer (*Hypothenemus hampei*) in Southern Mexico: a survey for a biocontrol project. Tropical Pest management, 35 (2):163-168.

BARRERA, J. F.; GÓMEZ, J.; ALAUZET, C. 1995. Can the coffee berry borer (*Hypothenemus hampei*) reproduce by parthenogenesis ?. Entomol. Exp. Appl. 77: 351-354.

BEILLE, L. 1925. Les stephanoderes sur les caféiers cultivés à la Côte d'Ivoire. Revue de Botanique Appliquée et d'Agriculture Tropicale 5: 387-388.

BENAVIDES, P.; 2005a. Aspectos genéticos de la broca del café, *Hypothenemus hampei*. *In*: Memorias XXXII Congreso de la Sociedad Colombiana de Entomología, Socolen, Ibagué (Colombia), Julio 27-29, 2005, p. 23-26.

BENAVIDES, P.; 2010a. ¿Cómo se dispersa la broca a partir de cafetales zoqueados?. Cenicafé, Brocarta No. 38, Chinchiná, Colombia, 2p.

BENAVIDES, P.; 2010b. Evite la dispersión de la broca durante la recolección y el beneficio del café. Cenicafé, Brocarta No. 40, Chinchiná, Colombia, 2p.

BENAVIDES, P.; BUSTILLO, A. E.; MONTOYA, E. C. 1994. Avances sobre el uso del parasitoide *Cephalonomia stephanoderis* para el control de la broca del café, *Hypothenemus hampei*. Revista Colombiana de Entomología, 20 (4): 247-253.

BENAVIDES, P.; BUSTILLO, A. E; MONTOYA, E. C.; CÁRDENAS, R.; MEJÍA, C. G. 2002. Participación del control cultural, químico y biológico en el manejo de la broca del café. Revista Colombiana de Entomología, 28 (2): 161-166.

BENAVIDES, P.; VEGA, F. E.; ROMERO-SEVERSON, J.; BUSTILLO, A. E.; STUART, J. 2005. Biodiversity and biogeography of an important inbred pest of coffee, coffee berry borer (Coleoptera: Curculionidae: Scolytinae). Annals Entomological Society of América, 98 (3): 359 - 366.

BENAVIDES, P.; VEGA, F.E.; ROMERO S., J.; BUSTILLO P., A.E.; STUART, J.J. 2005. Biodiversity and biogeography of an important inbred pest of coffee, coffee berry borer (Coleoptera: Curculionidae: Scolytinae). Annals of the Entomological Society of America (Estados Unidos) 98(3):359-366. 2005.

BENAVIDES, P.;. 2005. Distribución global de la broca del café: la versión molecular. *In*: Memorias XXXII Congreso de la Sociedad Colombiana de Entomología, Socolen, Ibagué (Colombia), Julio 27-29, 2005, p. 7-11

BERGAMIN, J. 1943. Contribuição para o conhecimento da biologia da broca do café *Hypothenemus hampei* (Ferrari, 1867) (Col. Ipidae). Arquivos do Instituto Biologico, São Paulo, 14: 31-72.

BERGAMIN, J. 1946. A broca do cafe no Brasil. Boletim da Superintendencia dos Servicos do Cafe (Brasil) 33:21-22

BERGAMIN, J.; KERR, WE. 1951. Determinação do sexo e citologia da broca do café. Ciencia Cultura 3: 117–121.

BERNAL, M. G.; BUSTILLO, A. E; POSADA, F. J. 1994. Virulencia de aislamientos de *Metarhizium anisopliae* y su eficacia en campo sobre *Hypothenemus hampei*. Revista Colombiana de Entomología, 20 (4): 225-228.

BORBON, O. 1990. Pérdidas de café provocadas por la broca del fruto del cafeto en Togo *Hypothenemus hampei* (Ferr). In: PROMECAFE (ed) IV Taller Regional sobre la Broca del Fruto del Cafeto. PROMECAFE, San Salvador (El Salvador)

BOUCIAS, D.; STOKES, C.; SUAZO, A.; FUNDERBURK, J. 2000. AFLP analysis of the entomopathogen *Nomuraea rileyi*. Mycologia 92: 638-648.

BREILID, H., L. O. BRUN, D. ANDREEV, R. H. FFRENCH-CONSTANT, AND L. KIRKENDALL. 1997. Phylogeographic patterns of introduced populations of the coffee berry borer *Hypothenemus hampei* (Ferrari)(Coleoptera: Scolytidae) inferred from mitochondrial DNA sequences, ASIC Colloque, Nairobi.

BRUN, L. O.; MARCILLAUD, C.; GAUDICHON, V.; SUCKLING, D. M. 1989. Endosulfan resistance in *Hypothenemus hampei* (Coleoptera: Scolytidae) in New Caledonia. Journal of Economic Entomology, 82 (5): 1312-1316.

BRUN, L. O.; STUART, J.; GAUDICHON, V.; ARONSTEIN, K.; FFRENCH-CONSTANT, R. H. 1995. Functional haplodiploidy: a mechanism for the spread of insecticide resistance in an important international insect pest. Proceedings National Academy of Sciences, (USA), 92: 9861-9865.

BUGNICOURT, F. 1950. Le scolyte du grain du café en Nouvelle-Caledonie. Rev. Agric. Nouv.-Cald. 1: 3-4.

BUSTILLO, A. E. 1991. Perspectivas de manejo integrado de la broca del café, *Hypothenemus hampei* en Colombia. Sociedad Colombiana de Entomología, Socolen, Medellín, Colombia. Miscelánea No. 18, p. 106-118.

BUSTILLO, A. E. 1995. Utilización del control biológico clásico en un programa de manejo integrado: El caso de la broca del café, *Hypothenemus hampei,* en Colombia. *In*: Memorias Curso Internacional Manejo Integrado de Plagas, ICA- Universidad de Nariño, nov. 27-dic. 1, 1995, San Juan de Pasto, Colombia, p. 143-148

BUSTILLO, A. E. 2002. El manejo de cafetales y su relación con el control de la broca del café en Colombia. FNC - Cenicafé, Chinchiná, Colombia. Boletín Técnico No. 24, 40 p.

BUSTILLO, A. E. 2004. ¿Cómo participa el hongo *Beauveria bassiana* en el manejo integrado de la broca del café?. Cenicafé, Brocarta No. 37, Chinchiná, Colombia, 4 p.

BUSTILLO, A. E.; CARDENAS, R.; POSADA, F. J. 2002. Natural enemies and competitors of *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae) in Colombia. Neotropical Entomology, 31 (4): 635 – 639.

BUSTILLO, A. E.; CASTILLO, H.; VILLALBA, D.; MORALES, E.; VÉLEZ, P. 1991. Evaluaciones de campo con el hongo *Beauveria bassiana* para el control de la broca del café, *Hypothenemus hampei* en Colombia. ASIC, 14e. Colloque, San Francisco, U.S.A., p. 679-686.

BUSTILLO, A. E.; MARIN, P. 2002. ¿Cómo reactivar la virulencia de *Beauveria bassiana* para el control de la broca del café?. Hoja Técnica No. 40. Catie. Revista Manejo Integrado de Plagas, No. 63. p. i– iv.

BUSTILLO, A. E.; OROZCO, J.; BENAVIDES, P.; PORTILLA, M. 1996. Producción masiva y uso de parasitoides para el control de la broca del café, *Hypothenemus hampei*, en Colombia. Revista Cenicafé (Colombia), 47 (4): 215-230.

BUSTILLO, A. E.; POSADA, F. J. 1996. El uso de entomopatógenos en el control de la broca del café en Colombia. Manejo Integrado de Plagas (Costa Rica), 42: 1-13.

BUSTILLO, A. E.; VILLALBA, D.; OROZCO J.; BENAVIDES, P.; REYES, I. C.; CHÁVES, B. 1995. Integrated pest management to control the coffee berry borer, *Hypothenemus hampei*, in Colombia. ASIC, 16e. Colloque, Kyoto, Japan, p. 671-680.

BUSTILLO, A. E; CÁRDENAS, R.; VILLALBA, D.; BENAVIDES, P.; OROZCO, J.; POSADA F. 1998. Manejo integrado de la broca del café, *Hypothenemus hampei* (Ferrari) en Colombia. Chinchiná, Cenicafé, Editorial Ferisa, Cali, Colombia, 134 p.

BUSTILLO, A.E. 2006. Una revisión sobre la broca del café, *Hypothenemus hampei* (Coleoptera: Curculionidae. Scolytidae), en Colombia. *Revista Colombiana de Entomología,* 32, 101-116.

CÁRDENAS, A. B.; VILLALBA, D. A.; BUSTILLO, A. E.; MONTOYA, E. C.; GÓNGORA, C. E. 2007. Eficacia de mezclas de cepas del hongo *Beauveria bassiana* (Bálsamo) Vuillemin en el control de la broca del café. Revista Cenicafé (Colombia), 58(4):293-303.

CÁRDENAS, R. 1983. La arañita roja del cafeto, *Oligonychus yothersi* McGregor. Cenicafé, Avances Técnicos No. 110, Chinchiná, Colombia, 2 p.

CÁRDENAS, R. 1985.La palomilla de las ramas del cafeto *Planococcus citri* (Risso) (Homoptera: Pseudococcidae.). Cenicafé, Avances Técnicos, No. 125, Chinchiná, Colombia, 2 p.

CASTAÑO, A.; BENAVIDES, P.; BAKER, P. S. 2005. Dispersión de *Hypothenemus hampei* en cafetales zoqueados. Revista Cenicafé (Colombia), 56 (2):142-150.

CASTILLO, A.; INFANTE, F.; BARRERA, J. F.; CARTA, L.; VEGA, F. E. 2002. First field report of a nematode (Tylenchida: Sphaerularioidea) attacking the coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae) in the Americas. Journal of Invertebrate Pathology, 79: 199–202.

CASTRILLO, L.A.; WIEGMANN, B.M.; BROOKS, W.M. 1998. Genetic variation in *Beauveria bassiana* populations associated with the darlkling beetle, *Alphitobius diaperinus.* J Invertebr Pathol 73: 269-275.

CASTRO, L.; BENAVIDES M., P.; BUSTILLO P., A.E. Dispersión y mortalidad de *Hypothenemus hampei*, durante la recolección y beneficio del café. Manejo Integrado de Plagas (Costa Rica) No. 50:19-28. 1998.

CENICAFÉ. 2010. Anuario Meteorologico Cafetero 2010. Chinchina, Colombia.

CHAMORRO, T. G.; CARDENAS, R.; HERRERA, H. A. 1995. Evaluación económica y de la calidad en taza del café proveniente de diferentes sistemas de recolección manual, utilizables como control en cafetales infestados de *Hypothenemus hampei*. Revista Cenicafé (Colombia), 46(3): 164-175.

COATES, .B.; HELLMICH, R.L.; LEWIS, L.C. 2002. Allelic variation of a *Beauveria bassiana* (Ascomycota: Hypocreales) minisatellite is independent of host range and geographic origin. Genome 45: 125-132.

CORBETT, G. H. 1933. Some preliminary observations on the coffee berry beetle borer *Stephanoderes (Cryphalus) hampei* Ferr. Malayan Agricultural Journal (Malaya), 21 (1): 8-22.

CRUZ, L.P.; GAITAN, A.L.; GÓNGORA, C.E. 2006. Exploiting the genetic diversity of beauveria bassiana for improving the biological control of the coffee berry borer through the use of strain mixtures. Appl Microbiol Biotechnol 71 (6):918-926. doi:10.1007/s00253-005-0218-0

DE LA ROSA, W.; ALTORRE, R.; BARRERA, J.F.; TORRELLO, C. 2000. Effect of *Beauveria bassiana* and *Metarhizium anisopliae* (Deuteromycetes) upon the coffee berry borer (Coleoptera: Scolytidae) under field conditions. *Journal of Economic Entomology*, 93, 1409 –1414.

DECAZY, B. 1987. Control de la Broca del fruto del cafeto *Hypothenemus hampei* Congreso Sobre el Cultivo del Cafe. ISIC-AIDE, San Salvador, El Salvador

DECAZY, B. 1990a. Descripción, biología, ecología y control de la broca del fruto del cafeto, *Hypothenemus hampei* (Ferr.). *In*: 50 años de Cenicafé 1938-1988, Conferencias conmemorativas. Chinchiná, Colombia, p.133-139.

DECAZY, B. 1990b. Niveles y umbrales de daños económicos de las poblaciones de la broca del fruto del cafeto, *Hypothenemus hampei* (Ferr.). *In*: 50 años de Cenicafé, 1938-1988, Conferencias conmemorativas, p.146-149.

DECAZY, B. 1990c. Métodos de muestreo para la determinación de poblaciones críticas de la broca del fruto del cafeto *Hypothenemus hampei* (Ferr.). En*:* 50 años de Cenicafé 1938-1988, Conferencias conmemorativas, Chinchiná, Colombia, p.140-145.

DIAZ, Y.; MARÍN, H. F. 1999. Evaluación de los frutos de café dejados después de las recolecciones durante un ciclo productivo del cultivo en dos municipios del Departamento de Caldas. Universidad de Caldas, Facultad de Ciencias Agropecuarias, (Tesis Ingeniero Agrónomo), Manizales, Colombia, 96 p.

DUQUE O., H. ; BAKER, P.S. Devouring profit; the socio-economics of coffee berry borer IPM. Chinchiná, *The CommoditiesPress* - CABI-CENICAFÉ, 2003. 105 p.

ECHEVERRY, O. A. 1999. Determinación del impacto de *Phymastichus coffea* La Salle (Hymenoptera: Eulophidae) sobre poblaciones de broca del café *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae), en la zona cafetera. Universidad Nacional de Colombia, Facultad de Ciencias Agropecuarias, (Tesis Ingeniero Agrónomo), Palmira, Colombia, 113 p.

EL CAFÉ DE NICARAGUA. 1978. Alarma en México: aparece la broca del café. El Café de Nicaragua, Nicaragua, p 14

EL CAFÉ DE NICARAGUA. 1979. El Café de Nicaragua Insectos y ácaros del follaje. El Café de Nicaragua (Nicaragua), Nicaragua, p 4-10

FFRENCH-CONSTANT, R. H.; STEICHEN, J. C.; BRUN, L. O. 1994. A molecular diagnostic for resistance in the coffee berry borer *Hypothenemus hampei* (Coleoptera: Scolytidae). Bulletin Entomological Research, 84: 11-16.

FLÓREZ, E.; BUSTILLO, A. E.; MONTOYA, E. C. 1997. Evaluación de equipos de aspersión para el control de *Hypothenemus hampei* con el hongo *Beauveria bassiana.* Revista Cenicafé (Colombia), 48 (2): 92- 98.

FNC. Federación Nacional de Cafeteros de Colombia. 2007. Informe anual de Gerencia Técnica, Sanidad Vegetal. Bogotá, Colombia, 3 p.

GAITAN, A.; VALDERRAMA, A.; SALDARRIAGA, G.; VELEZ, P.; BUSTILLO, A. E. 2002. Genetic variability of *Beauveria bassiana* associated with the coffee berry borer *Hypothenemus hampei*. Mycological Research, 106 (11): 1307 – 1314.

GAVIRIA, A. H.; CÁRDENAS, R.; MONTOYA, E. C.; MADRIGAL, A. 1995. Incremento poblacional de la broca del café *Hypothenemus hampei* relacionado con el desarrollo del fruto del cafeto. Revista Colombiana de Entomología, 21 (3): 145-151.

GIRALDO, D. P. 2003. Comportamiento de entomonematodos en el control de poblaciones de broca en árboles de café. Tesis Ingeniero Agrónomo. Facultad de Ciencias Agropecuarias. Universidad de Caldas. Manizales, Colombia, 83 p.

GLARE, T.R.; INWOOD, A.J. 1998. Morpohological and genetic characterization of *Beauveria* spp. from New Zealand. Mycol Res 102: 250-256.

GOETTEL, M.S.; EILENBERG, J.; GLARE, T. 2005. Entomopathogenic fungi and their role in regulation of insect populations. *In: Comprehensive Molecular Insect Science*, eds. K. Iatrou, L. Gilbert, and S. Gill, Amsterdam: Pergamon Press, pp. 361 – 405.

GÓNGORA, B.; POSADA, F. J.; BUSTILLO, A. E. 2001. Detección molecular de un gen de resistencia al insecticida endosulfan en una población de broca *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae) en Colombia. *In*: Resúmenes Congreso de la Sociedad Colombiana de Entomología, XXVIII, Socolen, Pereira, Colombia, agosto 8-10, 2001, p. 47-48

GÓNGORA, C. 2005. Avances en conocimiento y mejoramiento del hongo entomopatógeno *Beauveria bassiana* para el control de la broca del café, *Hypothenemus hampei*). *In*: Memorias XXXII Congreso Sociedad Colombiana de Entomología, Socolen, Ibagué, 27 – 29 de julio 2005, p. 27 – 32.

GÓNGORA, C. E.; WANG, C. E.; BARBEHENN, R. V.; BROADWAY, R. M.. 2000. Chitinolytic enzymes from *Streptomyces albidoflavus* expressed in tomato plants: effects on *Trichoplusia ni*. Entomologia Experimentalis et Applicata, 99: 193 – 204.

GÓNGORA, C.E.; MARÍN, P.; BENAVIDES, P. 2009. Claves para el éxito del hongo *Beauveria bassiana* como controlador biológico de la broca del café. Avance técnico 348, Cenicafé.

GONZÁLEZ, M. T.; POSADA, F. J.; BUSTILLO, A. E. 1993. Desarrollo de un bioensayo para evaluar la patogenicidad de *Beauveria bassiana* sobre *Hypothenemus hampei*. Revista Cenicafé (Colombia), 44(3): 93-102.

GOWDEY, C. C. 1910. Annual Report, pp. 7. Government Entomologist, Uganda.

GRESSEL, J. 2007. Failsafe mechanisms for preventing gene flow and organism dispersal of enhanced microbial biocontrol agents. 2007 In: Vurro M, Gressel J, eds. *Novel Biotechnologies for biocontrol agent enhancement and management*. Netherlands: Springer: pp. 353-362.

GRESSEL, J.; MEIR, S.; HERSCHKOVITZ, Y. *et al.* 2007. Approaches to and successes in developing transgenically enhanced mycoherbicides. In: Vurro M, Gressel J, eds. *Novel Biotechnologies for biocontrol agent enhancement and management.* Netherlands: Springer. pp. 277-296.

GUHARAY, F.; MONTERREY, J. 1997. Manejo ecológico de la broca del cafeto *Hypothenemus hampei* en América Central. Manejo Integrado de Plagas (Costa Rica) 45:i-viii

HAGEDORN, M. 1910. Wieder ein neuer kaffeeschädlinge. Entomologische Blätter 6: 1-14.

HARAPRASAD , N.; NIRANJANA, S. R.; PRAKASH, H.S.; SHETTY, H. S.; WAHAB S. 2001. *Beauveria bassiana* -A Potential Mycopesticide for the Efficient Control of Coffee Berry Borer, Hypothenemus hampei (Ferrari) in India. *Biocontrol Science and Technology*, 11, 25-260.

HERNÁNDEZ, P.; SÁNCHEZ, L. 1978. La broca del fruto del cafe. Guatemala. Revista Cafetalera (Guatemala) 174:.11-26.

ICA. INSTITUTO COLOMBIANO AGROPECUARIO. 1989. Lista de insectos dañinos y otras plagas en Colombia. Bol. Técnico No. 43, 4a ed., 662 p.

JARAMILLO, J.; BUSTILLO, A. E.; MONTOYA, E. C. 2002. Parasitismo de *Phymastichus coffea* sobre poblaciones de *Hypothenemus hampei* en frutos de café de diferentes edades. Revista Cenicafé, 53 (4): 317-326.

JARAMILLO, J.; BUSTILLO, A. E.; MONTOYA, E. C.; BORGEMEISTER, C. 2005. Biological control of the coffee berry borer *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae, Scolytinae) by *Phymastichus coffea* LaSalle (Hymenoptera: Eulophidae) in Colombia. Bulletin of Entomological Research, 95: 1 – 6.

JARAMILLO, J.; CHABI-OLAYE, A.; KAMONJO, C.; JARAMILLO, A.; VEGA, F.E.; POEHLING, H.; BORGEMEISTER, C. 2009. Thermal Tolerance of the Coffee Berry Borer *Hypothenemus hampei*: Predictions of Climate Change Impact on a Tropical Insect Pest. PLoS ONE 4(8): e6487.

JIMÉNEZ, J. A. 1992. Patogenicidad de diferentes aislamientos de *Beauveria bassiana* sobre la broca del café. Revista Cenicafé, 43 (3): 84-88.

JOHNSTON, A. 1963. Stephanoderis hampei in Tahiti, pp. 4 pp. Plant Protection Committee for the South East Asia and Pacific Region. Food and Agriculture Organization of

the United Nations, Regional Office for Asia and the Far East. Information Letter No. 23, Bangkok, Thailand.

JONG, S.N.; LÉVESQUE, C.A.; VERKLEY, J.M.; ABELN, C.A.;RAHE, J.E.; BRAUN, P.G. 2001. Phylogentic relationships among *Neofabraea* species causing tree cankers and bull's-eye rot of apple based on DNA sequencing of ITS nuclear rDNA, mitochondrial rDNA, and the β-tubulin gene. Mycol Res 105: 658-669.

LARA, J. C.; LÓPEZ, J. C. 2005. Evaluación de diferentes equipos de aspersión para la aplicación de nematodos entomopatógenos. Revista Colombiana de Entomología, 31 (1): 1 - 4.

LARA, J. C.; LÓPEZ, J. C.; BUSTILLO, A. E. 2004. Efecto de entomonematodos sobre poblaciones de la broca del café, *Hypothenemus hampei* (Coleoptera: Scolytidae), en frutos en el suelo. Revista Colombiana de Entomología, 30 (2): 179-185.

LE PELLEY, R. H. 1968. Pests of coffee. Longmans, Green and Co. Ltd., London, 590 p.

LEAL-BERTIOLI, S.C.M.; BUTT, T.M.; PEBERDY, J.F.; BERTIOLLI, D.J. 2000. Genetic exchange in *Metarhizium anisopliae* strains co-infecting *Phaedon cochleariae* is revealed by molecular markers. Mycol Res 104: 409-414.

LEPLAE, E. 1928. Le scolyte des baies du caféier (Stephanoderes). Bulletin Agricole du Congo Belge 19: 271-276.

LÓPEZ, J. C. 2002. Nematodos parásitos de insectos y su papel en el control de la broca del café, *Hypothenemus hampei* (Ferrari). En*:* Memorias Curso Internacional Teórico – Práctico. Sección II. Parasitoides y otros enemigos de la broca. Cenicafé, Chinchiná, Colombia, marzo 18 al 22, 2002, p. 39–70.

LÓPEZ, J. C.; CANO, L.; GÓNGORA C. E.; STOCK, S. P. 2007. Diversity and evolutionary relationships of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) from the Central Andean region of Colombia. Nematology, 9 (3): 333-341.

LÓPEZ, J. C.; GÓNGORA, C. E.; PLICHTA, K.; STOCK, P. PLICHTA K. 2008. A new entomopathogenic nematode, *Steinernema colombiense* n. sp. (Nematoda: Steinernematidae) from Colombia. Holanda Nematology, 10(4):561-574.

LÓPEZ-VAAMONDE, C.; BAKER, P. S.; COCK, M. J. W; OROZCO, J. 1997. Dossier on *Phymastichus coffea* (Hymenoptera: Eulophidae, Tetrastichinae), a potential biological control agent for *Hypothenemus hampei* (Coleoptera: Scolytidae), in Colombia. CABI, IIBC, Ascot, UK & Cenicafé, Chinchiná, Colombia, 23 p.

MALDONADO, C.E.; BENAVIDES, P. Evaluación del establecimiento de *Cephalonomia stephanoderis* y *Prorops nasuta*, controladores de *Hypothenemus hampei*, en Colombia. Cenicafé (Colombia) 58(4):333-339. 2007.

MARIN, P.; BUSTILLO, A. E. 2002. Producción artesanal de hongos entomopatógenos para el control de insectos plagas. En: Memorias Curso Internacional Teórico – Práctico. Sección I. Entomopatógenos de la broca del café. Cenicafé, Chinchiná, Colombia, marzo 11 al 15 del 2002. p. 125 – 131.

MENDOZA, M.E. 1996. Evaluación del daño ocasionado por la Broca del Café *Hypothenemus hampei* Ferrari 1867 (Coleoptera: Scolytidae), en los primeros estados de desarrollo del fruto, en dos zonas cafeteras del departamento del Valle del Cauca. In: Socolen (ed) XXIII Congreso de la Sociedad Colombiana de Entomología. Socolen, Cartagena (Colombia)

MESTRE, A.; OSPINA, H. F. 1994a. Estabilización de la producción en las fincas cafeteras. Cenicafé, Avances Técnicos, No. 200. Chinchiná, Colombia, 4 p.

MESTRE, A.; OSPINA, H. F. 1994b. Manejo de los cafetales para estabilizar la producción en las fincas cafeteras. Cenicafé, Avances Técnicos, No. 201. Chinchiná, Colombia, 8 p.

MESTRE, A.; SALAZAR, J. N. 1995. Producción de cafetales establecidos con una y dos plantas por sitio. Cenicafé, Avances técnicos, No. 213, Chinchiná, Colombia, 2p.

MOLINA, J. P.; LÓPEZ, J. C. 2002. Desplazamiento y parasitismo de entomonematodos hacia frutos infestados con la broca del café, *Hypothenemus hampei* (Coleoptera: Scolytidae). Revista Colombiana de Entomología, 28 (2): 145 – 151.

MOLINA, J. P.; LÓPEZ, J. C. 2003. Supervivencia y parasitismo de nematodos entomopatógenos para el control de *Hypothenemus hampei* Ferrari (Coleoptera: Scolytidae). Boletín de Sanidad Vegetal. Plagas (España), 29: 523 – 533.

MONTERREY, J. 1991. La broca del café en Nicaragua I Reunión Intercontinental sobre Broca del Café, Tapachula, Chiapas, México: 28-30

MONTOYA, E.C. 1999. Caracterización de la infestación del café por la broca y efecto del daño en la calidad de la bebida. Cenicafé (Colombia) 50:245-258

MONTOYA, S.; CARDENAS, R. 1994. Biología de *Hypothenemus hampei* (Ferrari) en frutos de café de diferentes edades. Revista Cenicafé (Colombia), 45: 5-13.

MORALES, E.; CRUZ, F.; OCAMPO, A.; RIVERA, G.; MORALES, B. 1991. Una aplicación de la biotecnología para el control de la broca del café. *In*: Colloque Scientifique International sur le Café, 14. San Francisco. 14-19 Juillet 1991, Paris, ASIC. p. 521-526.

MORENO, D.; BUSTILLO, A. E.; BENAVIDES, P.; MONTOYA, E. C. 2001. Escape y mortalidad de *Hypothenemus hampei* en los procesos de recolección y beneficio del café en Colombia. Revista Cenicafé (Colombia), 52 (2): 111 – 116.

MUÑOZ, R. 1988. Muestreo en fincas para determinar la población de broca (*Hypothenemus hampei* Ferr.) y metodología para calcular el nivel de daño económico. IICA, Boletín de PROMECAFE No. 38, p.4-14.

MUÑOZ, R. 1989. Ciclo biológico y reproducción partenogenética del cafeto, *Hypothenemus hampei* (Ferr.). Turrialba, 39 (3): 415-421.

NAVARRO, L.; GONGORA, C.E.; BENAVIDES, P. 2010. Single nucleotide polymorphism detection at the *Hypothenemus hampei Rdl* gene by allele-specific PCR amplification with T_m-shift primers. Pesticide Biochemstry and Physiology. 97(3): 204-208.

NCA. NATIONAL ACADEMY OF SCIENCES. 1969. Insect- pest management and control. Principles of plant and animal pest control, vol. 3. Publication 1695, Washington, D. C., 508 p

OROZCO, J. 1995. Uso de parasitoides de origen africano para el control de la broca en Colombia. Memorias XXII Congreso de SOCOLEN. Bogotá, julio 26 - 28, p. 102-108.

OROZCO, J. 2002. Guía para la producción del parasitoide *Phymastichus coffea* para el control de la broca del café. CFC -Cenicafé – Cabi Commodities, ICO, Chinchiná, Colombia,19 p.

OROZCO, J.; ARISTIZÁBAL, L. F. 1996. Parasitoides de origen africano para el control de la broca del café. Cenicafé. Avances Técnicos No. 223. Chinchiná, Colombia, 4 p.

PADILLA, G. N.; BERNAL, M. G.; VÉLEZ, P. E.; MONTOYA, E. C. 2000. Caracterización patogénica y morfológica de aislamientos de *Metarhizium anisopliae* obtenidos de diferentes órdenes insectiles. Revista Cenicafé (Colombia), 51 (1): 28-40.

PAVA-RIPOL, M.; POSADA, F.; MOMEN, B.; WANG, C.; St. LEGER, R. 2008. Increased pathogenicity against coffee berry borer, *Hypothenemus hampei* (Coleoptera: Curculionidae) by *Metarhizium anisopliae* expressing the scorpion toxin (AaIT) gene. J Invertebr Pathol 99: 220-226

PERALTA, J. 1995. Diagnóstico de la labor de recolección y repase para el manejo de la broca del café *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae) por agricultores. Tesis: Ingeniero Agrónomo, Universidad Nacional de Colombia, Facultad de Ciencias Agropecuarias, Palmira, Colombia, 71 p.

PORTILLA, M.; BUSTILLO, A. E. 1995. Nuevas investigaciones en la cría masiva de *Hypothenemus hampei* y de sus parasitoides *Cephalonomia stephanoderis* y *Prorops nasuta*. Revista Colombiana de Entomología, 21 (1): 25-33.

PORTILLA, M.; STREETT, D. 2006. Nuevas técnicas de producción masiva automatizada de *Hypothenemus hampei* sobre la dieta artificial Cenibroca modificada. Cenicafé 57(1): 37-50.

POSADA, F. J.; BUSTILLO, A. E. 1994. El hongo *Beauveria bassiana* y su impacto en la caficultura Colombiana. Agricultura Tropical (Colombia), 31 (3): 97 - 106.

POSADA, F. J.; OSORIO, E.; VELÁSQUEZ, E. 2002. Evaluación de la patogenicidad de *Beauveria bassiana* sobre la broca del café empleando el método de la aspersión foliar. Revista Colombiana de Entomología, 28 (2): 139-144.

POSADA, F. J.; VILLALBA, D. A.; BUSTILLO, A. E. 2004. Los insecticidas y el hongo *Beauveria bassiana* en el control de la broca del café. Revista Cenicafé (Colombia), 55 (2): 136 - 149.

PROMECAFE. 2002. Boletin Promecafe, 3.

RABB, R. L.; GUTHRIE, F. E. 1970. Concepts of pest management. Proceedings of a conference held at North Carolina State University at Raleigh, March 25-27, 1970. 242 p.

REID, J.C. 1983. Distribution of the coffee berry borer *Hypothenemus hampei* within Jamaica, following its discovery in 1978. Trop. Pest Manage. 29:224-230

RIVERA, H. 1997. Establezca coberturas nobles en su cafetal utilizando el selector de arvenses. Cenicafé, Avances técnicos No. 235, Chinchiná, Colombia, 8 p.

RIVERA, H. 2000. El selector de arvenses modificado. Cenicafé, Avances técnicos No. 271, Chinchiná, Colombia, 4 p.

RODRIGUEZ, M. L.; GÓNGORA, C. E. 2005. Transformación de *Beauveria bassiana* cepa Bb9205 con los genes pr1A, pr1J y ste1 de *Metarhizium anisopliae* y evaluación de su patogenicidad sobre la broca del café. Revista Colombiana de Entomología, 31: 51-58.

ROMERO, H. 1990. La broca del café. Guia tecnica. Proyecto Agroyungas AD/BOL/84/405, p 25

ROSALES, M.; SILVA, R.; RODRIGUEZ, G. 1998. Estrategias para el manejo integrado del minador de la hoja y la broca del fruto del cafeto. FONAIAP Divulga (Venezuela):19-24

ROY, H.E.; STEINKRAUS, D.C.; EILENBERG, J.; HAJEK, A.E.; PELL, J.K. 2006. Bizarre Interactions and end Games: Entomopathogenic Fungi and Their Arthropod Hosts. *Annual Review of Entomology*, 51, 331–357.

RUALES, C. 1997. Aspectos generales sobre la broca del cafe en Ecuador. Cafe y Cacao: Noticias (Ecuador) 2:7-11

RUIZ, L.; BUSTILLO, A. E.; POSADA, F. J.; GONZÁLEZ M. T. 1996. Ciclo de vida de *Hypothenemus hampei* en dos dietas merídicas. Revista Cenicafé (Colombia), 47 (2): 77-84.

RUIZ, R. 1996. Efecto de la fenología del fruto del café sobre los parámetros de la tabla de vida de la broca del café; *Hypothenemus hampei* (Ferrari). Universidad de Caldas,

Facultad de Ciencias Agropecuarias, Manizales (Colombia), (Tesis: Ingeniero Agrónomo). 87 p.

SALAZAR, H. M.; BAKER, P. S. 2002. Impacto de liberaciones de *Cephalonomia stephanoderis* sobre poblaciones de *Hypothenemus hampei*. Revista Cenicafé (Colombia), 53 (4): 306-316.

SALDARRIAGA, G. 1994. Evaluación de prácticas culturales en el control de la broca del café *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae). Tesis Ingeniero Agrónomo, Universidad Nacional de Colombia, Facultad de Ciencias Agropecuarias, Medellín, Colombia, 57 p.

SCHMUTTERER, H. 1969. *Pests of crops in northeast and central Africa with particular reference to the Sudan.*,

SLININGER, P.J.; SCHISLER, D.A.; SHEA-ANDERSH , M.A.; SLOAN, J.M.; WOODELL, L.K.; FRAZIER, M.J.; OLSEN, N.L. 2010. Multi-strain co-cultures surpass blends for broad spectrum biological control of maladies of potatoes in storage. Biocontrol Science and Technology 20 (8):763-786. DOI:10.1080/09583151003717201

SPONAGEL, K. W. 1994. La broca del cafe, *Hypothenemus hampei* en plantaciones de Cafe Robusta en la Amazonia Ecuatoriana. Wissenschaftlicher Fachverlag, Giessen (Alemania).

SREEDHARAN, M.M.; BALAKRISHNAN, C.B.; PRAKASAN, P.; KRISHNAMOORTHY B.; NAIDU, R. 1994. Bio-ecology and management of coffee berry borer. Indian Coffee 58: 5-13.

ST. LEGER, R.; ALLEE, L.; MAY, B.; STAPLES, R.; ROBERTS, D. 1992. Worldwide distribution of genetic variation among isolates of *Beauveria* spp. Mycol Res 96: 1007-1015.

ST. LEGER, R.; WANG, S. 2010. Genetic engineering of fungal biocontrol agents to achieve greater efficacy against insect pests. Appl Microbiol Biotechnol 85(4):901-907. dOI: 10.1007/s00253-009-2306-z Stuttgart, Gustav Fischer Verlag:296pp.

TICHELER, J.H.G. 1963. Estudio analítico de la epidemiología del escolítido de los granos de café, *Stephanoderis hampei* Ferr., en Costa de Marfil (Traducción G. Quiceno). Revista Cenicafé (Colombia), 14 (4): 223-294.

TIGANO-MILANI, M.S.; HONEYCUTT, R.J.; LACEY, L.A.; ASSIS, R.; MCCLELLAND, M.; SOBRAL, B.W.S. 1995. Genetic variability of *Paecilomyces fumosoroseus* isolates revealed by molecular markers. J Invertebr Pathol 65: 274-282.

VALDERRAMA, A. M.; CRISTANCHO, M. A.; CHAVES B. 2000. Análisis de la variabilidad genética del hongo entomopatógeno *Beauveria bassiana* con marcadores RAPD. Revista Colombiana de Entomología, 26 (1-2): 25-30.

VALDÉS, B. E.; VÉLEZ, P. E; MONTOYA, E. C. 1999. Caracterización enzimática y patogenicidad de aislamientos de *Beauveria bassiana* sobre la broca del café. Revista Cenicafé (Colombia), 50 (2): 106-118.

VARAPRASAD, K. S.; BALASUBRAMANIAN, S.; DINAKAR, B. J.; RAO, R. C. V. R. 1994. First report of an entomogenous nematode, *Panagrolaimus* sp., from coffee-berry borer, *Hypothenemus gampei* (Ferrari) from Karnataka, India. Plant Protection Bulletin Faridabad, 46 (2-3): 34.

VEGA, F.; BENAVIDES, P.; STUART, J. J.; O'NEIL, S. 2002. *Wolbachia* infection in the coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae). Annals Entomological Society of America, 95 (3): 374-378.

VÉLEZ, M. 2002. Hormigas y su papel en el control biológico de la broca del café. En: Memorias Curso Internacional Teórico – Práctico. Sección II. Parasitoides y otros

enemigos de la broca del café. Cenicafé, Chinchiná, marzo 18 al 22 de 2002, p. 15 – 23.

VÉLEZ, P. E.; ESTRADA, M. N.; GONZÁLEZ, M. T.; VALDERRAMA, A. M.; BUSTILLO, A. E. 2001. Caracterización de aislamientos de *Beauveria bassiana* para el control de la broca del café. Manejo Integrado de Plagas (Costa Rica), 62: 38 -53.

VÉLEZ, P. E.; GONZÁLEZ, M. T.; RIVERA, A.; BUSTILLO, A. E.; ESTRADA, M. N.; MONTOYA, E. C. 1999. Caracterización de aislamientos de *Beauveria bassiana* y *Metarhizium anisopliae* de la colección de Cenicafé. Revista Colombiana de Entomología, 25 (3-4): 191-207.

VÉLEZ, P. E.; POSADA, F. J.; MARIN, P.; GONZÁLEZ, M. T.; OSORIO, E.; BUSTILLO, A. E. 1997. Técnicas para el control de calidad de formulaciones de hongos entomopatógenos. Boletín Técnico, No 17, Cenicafé, Chinchiná, Colombia, 37 p.

VÉLEZ, P.E., AND BENAVIDES, G.M. (1990), 'Registro e identificacio´n de Beauveria bassiana en Hypothenemus hampei en Ancuya, Departamento de Narin˜o, Colombia', Revista Cenicafe´,41, 50_57.

VERA M., L.Y.; GIL P., Z.N.; BENAVIDES M., P. Identificación de enemigos naturales de *Hypothenemus hampei* en la zona cafetera central colombiana. Cenicafé (Colombia) 58(3):185-195. 2007.

VERGARA, J. D.; OROZCO, J., BUSTILLO, A. E.; CHÁVES, B. 2001b. Dispersión de *Phymastichus coffea* en un lote de café infestado de *Hypothenemus hampei*. Revista Cenicafé (Colombia), 52 (2): 104 – 110.

VERGARA, J. D.; OROZCO, J.; BUSTILLO, A. E.; CHÁVES, B. 2001a. Biología de *Phymastichus coffea* en condiciones de campo. Revista Cenicafé (Colombia), 52 (2): 97-103.

VERNALHA, M. M.; S. G. SOARES, J. C. GABARDO, M. A. L. D. ROCHA, L. G. C. VELLOZO, M. J. NOWACKI, AND O. S. FONTURA. 1965. Pragas e doencas do cafeeiro no estado do Parana. Universidade Federal do Parana, Parana (Brasil).

VILLALBA, D. A.; BUSTILLO, A. E.; CHÁVES, B. 1995. Evaluación de insecticidas para el control de la broca del café en Colombia. Revista Cenicafé (Colombia), 46 (3): 152-163

WANG, C.; FAN, M.; LI, Z.; BUTT, T.M. 2004. Molecular monitoring and evaluation of the application of the insect-pathogenic fungus *Beauveria Bassiana* in southeast China. J Appl Microbiol 96: 861-870.

WATTSON, D.W.; PETERSEN, J.J. 1993. Sexual activity of male *Musca domestica* (Diptera: Muscidae) infected with *Entomophthoramuscae* (Entomophthorales: Entomophthoraceae)', *Biological Control,* 3, 22–26.

WHITE, T.J.; BRUNS, T.; LEE, S.; TAYLOR, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR Protocols: A guide to methods and applications. Academic Press, San Diego, pp 315-322.

24

Electroanalysis of Insecticides at Carbon Paste Electrodes with Particular Emphasis on Selected Neonicotinoid Derivatives

Valéria Guzsvány[1], Zsigmond Papp[1], Ivan Švancara[2] and Karel Vytřas[2]
[1]*Department of Chemistry, Biochemistry and Environmental Protection, Faculty of Sciences, University of Novi Sad, Novi Sad*
[2]*Department of Analytical Chemistry, Faculty of Chemical Technology, University of Pardubice, Pardubice*
[1]*Republic of Serbia*
[2]*Czech Republic*

1. Introduction

1.1 Recent trends in the use of insecticides

Insecticides are substances from the group of pesticides intended for preventing, destroying, repelling or mitigating insects (Pesticide, 2011). Although there are benefits to the use of insecticides, there are also drawbacks, such as potential toxicity to humans and other animals. Residues in fruit and vegetables, cereals, processed baby food and foodstuffs of animal origin are controlled through a system of statutory maximum residue limits (MRLs) (Tuzimski, 2011).

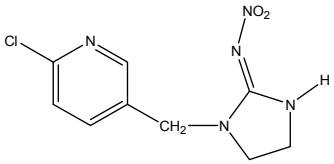
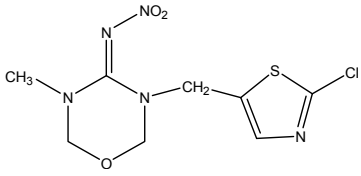
The increasing use of pesticides, especially herbicides and insecticides, in agriculture, forestry, and domestic activities for controlling pests causes pollution of the water resources, environment, as well as of many food stuff. The leaching run-off from agricultural and forest lands; deposition from aerial applications and residua from the industrial wastewater treatment are mainly responsible for the water contamination (Gupta, 2004). The pesticides form a strong class of water and environment pollutants, as they are sometimes non biodegradable. The toxicity of pesticides and their degradation products make these chemical substances potentially hazardous contaminants of the environment (Schultz et al., 2003). According to the Stockholm Convention on Persistent Organic Pollutants, nine of the dozen of the most harmful and persistent organic chemicals are pesticides (Ridding the World of POPs: A guide to the Stockholm Convention on Persistent Organic Pollutants, 2005; Pesticide, 2011).

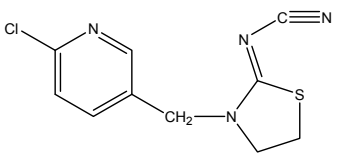
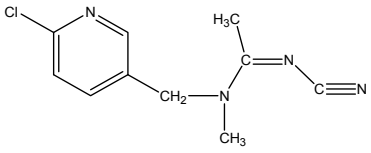
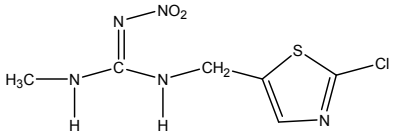
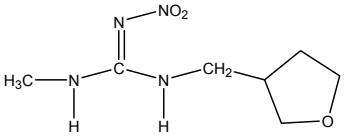
The insecticides can be grouped by means of sorting into chemical families. Major insecticide families include organochlorines, organophosphates, carbamates, and neonicotinoids. Organochlorine hydrocarbons (e.g. DDT) could be separated into dichlorodiphenylethanes, cyclodiene compounds, and other related compounds. They operate by disrupting the sodium/potassium balance of the nerve fiber, forcing the nerve to transmit continuously. Their toxicities vary greatly, but they have been phased out because of their persistence and potential to bioaccumulate (Kamrin, 1997). For instance, due to

extreme stability of highly toxic organochlorines, these formerly popular products (like the above-mentioned DDT) have largely been replaced by organophoshates and carbamates. Nevertheless, they are toxic as well, operating through inhibition of the enzyme acetylcholinesterase, allowing acetylcholine to transfer nerve impulses indefinitely and causing a variety of symptoms such as weakness or paralysis. Moreover, organophosphates are quite toxic to vertebrates, and have to be, in some cases, been replaced by less toxic carbamates (Kamrin, 1997), or by thiocarbamates and dithiocarbamates as the subclasses of carbamates.

Thus, there was a demand for less harmful compounds, which would become the case of **neonicotinoid insecticides** whose successful story had started in 1991 with the launching of a forerunner, *Imidacloprid*, by Bayer Crop Science, being the world's largest selling insecticide for many years (Nauen et al., 2008). Today, neonicotinoids (see **Table 1**), acting on the level of nicotinic acetylcholine receptor (nAChR) of insects, are one of the most important categories of insecticides introduced to the global market since the synthetic pyrethroids (Jeschke & Nauen, 2008; Jeschke et al., 2011). Up until now, neonicotinoids are the most important class of insecticides introduced to the global market since the synthetic pyrethroids, when the former are registered globally in more than 120 countries, regarded as the most effective insecticides to control sucking insect pests such as aphids, whiteflies, leaf and planthoppers, thrips, some micro-Lepidoptera, and a number of coleopteran pests (Jeschke & Nauen, 2008).

The outstanding development of neonicotinoid insecticides for modern crop protection, consumer / professional products, and animal health markets in the last two decades reflects the enormous importance of this chemical class. In 1990, before the launch of the first neonicotinoid insecticide, imidacloprid, the agrochemical market (total volume of ca. eight billion EUR was dominated by organophosphates (OPs) (43%), pyrethroids (18%), and carbamates (16%). In 2008, the neonicotinoids had gained a 24% share of a slightly decreased total market of €6.330 billion, mainly at the expense of OPs (13.6%) and carbamates (10.8%) (see internal data from *Bayer Crop Science* in Jeschke et al., 2011). As a logical step, massive and still expanding application of neonicotinoids requires also new analytical measurements that can be operated in very diverse samples, including recently demonstrated monitoring of photodegradation process(es).

Trivial name	IUPAC name	Structural formula	Typical use
Imidacloprid	(*E*)-1-(6-chloro-3-pyridylmethyl)--*N*-nitroimidazolidin--2-ylideneamin		Applied by soil or tree injection, or in granular form. Effective to protect maize, sunflower
Thiamethoxam	3-(2-chloro-1,3-thiazol-5-ylmethyl)-5-methyl-1,3,5-oxadiazinan-4--ylidene(nitro)amine		Effective against aphids, thrips, beetles, centipedes, millipedes, sawflies, leaf miners, stem borers and termites

Trivial name	IUPAC name	Structural formula	Typical use
Thiacloprid	(*Z*)-3-(6-chloro-3-pyridylmethyl)--1,3-thiazolidin-2--ylidenecyanamide		Applied by soil or tree injection, or in granular form. Effective against sucking and chewing insects, primarily aphids and whiteflies
Acetamiprid	(*E*)-N^1-[(6-chloro-3--pyridyl)methyl]--N^2-cyano-N^1-methylacetamidine		Effective to control sucking insects on crops such as leafy vegetables, citrus fruits, pome fruits, grapes, cotton, cole crops, and ornamental plants. It is also a key pesticide in commercial cherry farming due to its effectiveness against the larvae of the cherry fruit fly.
Clothianidin	(*E*)-1-(2-chloro-1,3-tiazol-5-ylmethyl)-3-methyl-2--nitroguanidine		Effective to seed treatment for corn and canola
Dinotefuran	(*RS*)-1-methyl-2-nitro-3-(tetrahydro-3--furylmethyl)guanidine		Effective for the control of insect pests such as aphids, whiteflies, thrips, leafhoppers, leafminers, sawflies, mole cricket, white grubs, lacebugs, billbugs, beetles, mealybugs, and cockroaches on leafy vegetables, in residential and commercial buildings, and for professional turf management

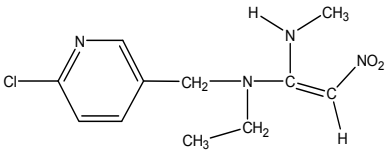
Trivial name	IUPAC name	Structural formula	Typical use
Nitenpyram	(*E*)-*N*-(6-chloro-3-pyridylmethyl)--*N*-ethyl-*N*′-methyl-2-nitrovinylidenediamine		Used in agriculture and veterinary medicine to kill insect external parasites of livestock and pets

Table 1. The commercial neonicotinoid insecticides (assembled according to Jeschke et al., 2011).

Besides a great number of benefits that these insecticides have, it is important to note that there is a hypothesis that neonicotinoid insecticides used for seed coating of agricultural crops – mainly corn, sunflower and seed rape – are related to the extensive death of honey bees. The death of honey bees, *Apis mellifera L.*, and the consequent colony collapse disorder causes major losses in agriculture and plant pollination worldwide. The phenomenon showed increasing rates in the period of 2008-2010, although its causes are still awaiting a clear answer. Many hypotheses, such as infections of parasitic mites (Cox-Foster et al. 2007), viruses (Anderson and Gibbs, 1988), chronic exposure to sub-lethal doses of insecticides (Desneux et al., 2007; Yang et al., 2008; Johnson et al., 2009; Alaux et al., 2010) or acute effects of neonicotinoid insecticides (Suchail et al., 2000) were formulated to account for bee decline. Although neonicotinoid systemic insecticides used for seed coating of agricultural crops were suspected as possible reason, studies so far have not shown the existence of unquestionable sources capable of delivering directly intoxicating doses in the fields. Guttation is a natural plant phenomenon causing the excretion of xylem fluid at leaf margins. Recently was found that leaf guttation drops of all the corn plants germinated from neonicotinoid-coated seeds contained amounts of insecticide constantly higher than 10 mg L^{-1}, with maxima up to 100 mg L^{-1} for thiamethoxam and clothianidin, and up to 200 mg L^{-1} for imidacloprid. The concentration of neonicotinoids in guttation drops can be near those of active ingredients commonly applied in field sprays for pest control, or even higher. When bees consume guttation drops, collected from plants grown from neonicotinoid-coated seeds, they encounter death within few minutes (Girolami et al., 2009; Tapparo et al., 2011).

1.2 Instrumental analysis of insecticides. A survey of contemporary trends

Due to the growing use of insecticides, their accumulation in the environment and foodstuff is evident. For these reasons, it has been necessary to develop sensitive analytical methods for monitoring the low levels of insecticide residues in soil, water, and agricultural products. The leading analytical techniques in the monitoring of insecticides in different complex matrices seem to be the techniques that combine separation and determination steps which allowed multiresidue, multiclass, and trace level analysis. Chromatographic techniques like gas chromatography (GC) and high performance liquid chromatography (HPLC) combined with different types of detectors, mainly mass spectrometric detector (MS) or tandem MS detector are on the leader position (Rocío et al., 2011; Feo et al., 2011). The choise of the chromatographic technique depends on the nature of investigated compound(s), first of all their polarity and basic-acid properties (Lambropoulou and Albanis, 2007), and the type of the sample matrix. In the case of GC in combination with MS, there is a database for the

identification of the insecticides from the samples. For the GC analysis advantage have the volatile compounds, but after their appropriate derivatisation in more volatile compound the low volatile or thermolabile compounds can be analysed, also. On the other hand, HPLC is recommended for the analysis of low volatile compounds and for compounds that are unstable when heated. Capillary electrophoresis (CE) and capillary electrochromatography (CEC) are convenient also for the determination of different target insecticides from different complex matrices (Cooper et al., 2000; Picó et al., 2003; Hernández-Borges et al., 2004; Lin et al., 2009). However, the chromatographic techniques are the most powerful tools for examining the contents of a very different insecticides in complex samples, these instruments are related primarily to laboratories, the samples (either liquid or solid samples) very often undergoes preliminary extractions before the chromatographic measurements, when their purchase- and operational costs are always considerably high.
Furthermore, there is a wide spectrum of alternative, mostly portable, non-chromatographic analytical equipments, first of all portable sensors, which are often used for the first screening of target analyte(s). Different techniques from molecular spectrometry (UV/Vis spectrophotometry, fluorimetry, colorimetry, infrared spectrometry), surface plasmone resonance sensors or immunochemical recognitions are used as screening techniques for the obtaining information about pesticide content in very different samples. The biorecognition elements can stay as independent techniques (e.g. Van Dyk and Pletschke, 2011 and the references herein), but they are often combined with other techniques, allowing higher specificity for identification and determination of the target analytes (Shankaran et al., 2007).
Regarding the neonicotinoid insecticides themselves, there is already a number of procedures employing various instrumental techniques and proposed for their detection and quantification in different samples. Often, such methods of choice are being based on high- and ultra-performance liquid chromatography (HPLC and UPLC) combined with the sensitive detection by a diode-array (Obana et al., 2002; Mandić et al., 2005; Watanabe et al., 2007), mass spectrometry (Obana et al., 2003; Kamel, 2010; Liu et al., 2010), thermal-lens spectrometry (Guzsvány et al., 2007a), amperometric detector (De Erenchun et al., 1997) or with an electrochemical detector and post column photochemical reactor (Rancan et al., 2006a,b). Furthermore, procedures based on photochemically induced fluorescence detection (Vilchez et al., 1998; Vilchez et al., 2001), micellar electrokinetic capillary chromatography (Carretero et al., 2003) and Fourier transform infrared spectrometry (Quintas et al., 2004), simple derivative spectrophotometry (Guzsvány, 2006; Guzsvány et al., 2009a) have also been used for the determination of imidacloprid or thiamethoxam. There are also studies related to bioassayes, the enzyme-linked immuno assay (ELISA) for identifying imidacloprid (Li et al., 2000; Lee et al., 2001; Kim et al., 2003; Watanabe et al., 2006), acetamiprid (Watanabe et al., 2006) and thiamethoxam (Kim et al., 2003; Kim et al., 2006). The above techniques are very convenient not just for the neonicotinoid residue analysis, or quality control of the commercial formulations, they are widely applied for the monitoring of the fate, stability and removal of these insecticides (Guzsvány, 2006; Guzsvány et al., 2008a,b; Guzsvány et al., 2009b; Guzsvány et al., 2010; Guzsvány et al., 2011b; Pena et al., 2011; Černigoj et al., 2008; Malato et al., 2001; Malato et al., 2002; Malato et al., 2003, and the references herein; Dell'Arciprete et al., 2010).

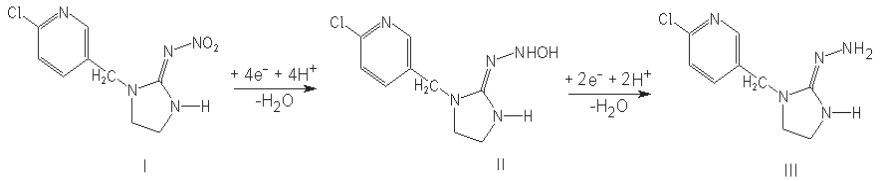
1.3 Electrochemistry and electroanalysis of insecticides

Electrochemical techniques, represent yet another alternative for the determination of various pollutants. Their main trump, compared to GC, HPLC, CE, CEC, and other

sophisticated instrumentations, is a fairly low cost and wide flexibility, including relatively easy adaptation for field monitoring (Wang, 2002). Among the electrode systems available, various sensors and biosensors based on heterogeneous carbon materials have always attracted considerable attention, which is also the case of procedures proposed exclusively for analysis of organic pollutants (see e.g. Ulakhovich, 1993; and refs. therein).

Perhaps the most effective approaches to analyse insecticides electrochemically are represented by voltammetric techniques; namely, **cyclic voltammetry** (CV) for investigations/elucidation of the reaction mechanisms (Gosser, Jr., 1993) and the-so-called **stripping voltammetry** combined with preconcentration (accumulation) step for the trace level determination of target analytes. Regarding the potential ramp, most frequently used are the differential pulse voltammetry (DPV) for its reliable performance at low concentration levels (Kissinger and Heineman, 1996) and — still more often — square-wave voltammetry (SWV) for its insensitivity to dissolved oxygen (Lovric, 2005). Exceptionally, for sufficiently high concentration ranges, one can choose also classical polarography with the dropping mercury electrode (Barek et al., 2001).

As shown during fundamental polarographic investigations (see e.g. Navalón et al., 1999; Guziejewski et al., 2011a,b; Guzsvány et al., 2006; with other refs. therein), the determination of nitroguanidine neonicotinoids can be based on the irreversible reduction of the electroactive nitro group to hydroxylamine and amine. For instance, when reducing *Imidacloprid* at the mercury electrode, one has:



(1)

The whole reduction pathway (**1**) with schemes I-III (redrawn after Navalón et al., 1999) is complex, depending on experimental conditions, when the single wave or even more consecutive responses may be obtained. Besides the potential applied, the process strongly depends upon the pH, whose variations are reflected in the overall peak current intensity, as well as the respective peak potentials that are being shifted to more negative range with the increasing pH. In this respect, the neonicotionoids behave as many other electroactive organic compounds and their precise identification in more complex samples requires the corresponding standards (see below, in sections 2 and 3). Best developed and sufficiently separated peaks of the neonicotinoids were mostly observed in neutral and slightly alkaline solutions that had also represented the supporting media of choice for analytical purposes.

Regarding this specific group of insecticides, the individual methods proposed for their determination have employed the mercury-based working electrodes (Navalón et al., 1999; Guiberteau et al., 2001; Guzsvány, 2006; Guzsvány et al., 2006a with other refs. therein; Guziejewski et al., 2011a,b) or detection units incorporating various unmodified (Guzsvány et al., 2005; Guzsvány, 2006; Guzsvány et al., 2007a; Guzsvány et al., 2008a; Papp et al. 2009a,b; Papp et al., 2010; Papp, 2011; Papp et al., 2011) and bismuth particles modified carbon based electrodes (Guzsvány, 2006; Guzsvány et al., 2006c; Gaál et al., 2007; Guzsvány et al., 2008a,b; Guzsvány et al., 2011a) as well as on copper(II) phthalocyanine modified carbon ceramic electrode (Majidi et al., 2011) or the nanosilver Nafion®/nanoTiO_2 Nafion® composite modified glassy carbon electrode (Kumaravel and Chandrasekaran, 2011).

1.4 A little excursion into the electrochemistry with carbon paste-based electrodes

Intro. Apparently, the most popular heterogeneous carbon electrodes are the so-called **carbon paste electrodes**, (CPEs), invented and largely propagated by Adams a half a century ago (Adams, 1958; Švancara et al., 2009a; Švancara et al., 2011). Carbon paste electrodes are applied worldwide; primarily, because of their broad potential window (mainly at the anodic side), very low residual currents (background), unique surface characteristics, low cost, simple preparation (directly in labs), usually minimal toxicity, and mainly, thanks to almost countless possibilities of their chemical and biological modifications (Kalcher K., 1990; Kalcher et al., 1995; Kalcher et. al, 2006; Švancara et al., 2011).

Common carbon pastes and their modified variants. In brief, the **bare** (binary, unmodified) **carbon paste electrode, CPE**, is an assembly of a mixture of graphite (carbon) powder, suitable liquid (binder), which is − as a soft and incompact material − packed into a convenient electrode body (Adams, 1963). Both main components, as well as their mutual ratio then co-determine the physicochemical and electrochemical properties that can further be altered – and often purposely controlled – by adding a third constituent, when the substance of such a choice can be a modifier / stabilizer / catalyst / mediator, etc. In these cases, one obtains **chemically** or **biologically modified carbon paste electrodes**, **CMCPEs** (Kalcher, 1990) and **CP-biosensors** (Gorton, 1995), respectively. Largely, bare carbon paste mixtures are being made from highly pure spectroscopic graphites (with mesh *ca.* 5-20 μm) and mineral oil (Nujol) or silicone oils and greases; all serving as a binder. Whereas these mixtures are called **traditional carbon pastes** (Kalcher et al., 1995; Kalcher et al., 2006), there is also a wide spectrum of other types being classified as special carbon pastes.

Special Carbon Pastes. Such mixtures with their basic characterisation have been of particular interest in some latest reviews - see *e.g.* Švancara et al., 2009b; Švancara et al., 2011 - showing that the pastes preparable from the alternate carbon paste constituents represent a long line of possible candidates. Thus, instead of graphite, one can e.g use glassy carbon globules, carbon fibres, carbon nanotubes, nanohorns, and fullerenes, as well as (pyrolysis-produced) carbon and acetylene black, template carbon, purified coal or even powdered (and specially doped) diamond. The proper replacement for traditional oily binders can then be some liquid organic esters, higher hydrocarbons, halogenated aromates, or room-temperature ionic liquids (RTILs); the latter being especially popular in the recent years.

Tricresyl phosphate-based carbon paste electrode (TCP-CPE). One of such special carbon pastes is also a mixture made of graphite and liquid tricresyl phoshate (TCP), introduced into the electrochemistry with CPEs in the early 1990s (Srey, 1992; Švancara and Vytřas, 1993). The making of the TCP-CPE was inspired by the research work by Kalcher who had used related substances as special liquid modifiers with distinct ion-exchange properties (Kalcher, 1985a,b; Kalcher et al., 1987), as well as its previous use as plasticizer of liquid membranes for ion-selective electrodes (Vytřas, 1985). The TCP-CPE is a typical representative of CPEs with electrochemically active binder, where the molecules of organophoshate can readily be protonated (Švancara et al., 1998a):

$$(C_7H_7O)_3P{=}O + H^+ \longrightarrow [(C_7H_7O)_3P{=}OH]^+ \quad (2a)$$

and, in this form, acting in numerous ion-exchange / ion-pairing processes; e.g.:

$$[(C_7H_7O)_3P{=}OH]^+ + Y^- \longrightarrow \{[(C_7H_7O)_3P{=}OH]^+ ; [Y]^-\} \quad (2b)$$

giving rise to the corresponding product – relatively stable and electroactive ion-associate.
Since then on, the TCP-CPE had successfully been employed in a number of electrochemical methods, utilising the above principles, given above in schemes (**2a**) and (**2b**) in combination with either voltammetric detection or potentiometric indication. More specifically, the TCP-CPE was the electrode of choice serving for the determination of gold in the form of $[AuCl_4]^-$ (Švancara and Vytřas, 1993; Vytřas et al., 1993), silver ions down to the picomolar level (Švancara et al., 1996b), bismuth as $[BiI_4]^-$ (Švancara and Vytřas, 1993), thallium via $[TlCl_4]^-$ (Konvalina and Vytřas, 1999), or numerous anions like BF_4^-, ClO_4^-, $HAsO_4^{2-}$, $[P(Mo_3O_{10})_4]^-$ (see Vytřas and Švancara, 2007 and refs. therein), and mainly for iodide (as I^- or I_3^- resp.; see e.g. Švancara et al., 1998a; Švancara et al., 2002). Moreover, the TCP-CPE could also be used for some theoretically oriented studies, which is the case of the microscopic study on the surface morfology of different carbon pastes (Švancara et al., 1996a), or the evaluation of stability constants for aurate(III) halides of the $[AuX4]^-$ type (where "X" is F, Cl, Br, SCN, and CN). Regarding organic substances and biologically important compounds, the tricresyl phosphate-based CPEs had thus far been tested in (preliminary) assays with polyaromatic nitrocompounds, PANs (Švancara et al., 1998b) and (unsuccessful) accumulation studies with 6-benzylaminopurine (a plant hormone; Švancara et al., 2001b). Thus, recent investigations on the applicability of the TCP-CPE in analysis of neonicotinoids, being in focus herein, represent just another of the few attempts to employ this rather unusual CPE in analysis of organic compounds and pollutants.
Bismuth-modified carbon paste electrodes. As mentioned above, the most appreciated feature of the CP-based electrode material is, without doubts, the fact that its electrochemical and electroanalytical value can immeasurably be enhanced by proper chemical (or biological) modification (Kalcher K., 1990; Kalcher et al., 1995; Kalcher et. al, 2006; Švancara et al., 2011). In the just-passed decade, one of the most intensively studied modification of CPEs and CMCPEs was their conversion into the **bismuth-**based **electrodes** and **sensors**, representing a new type of environmentally friendly detection system and, hand in hand, a principal step of modern electroanalysis into the realm of momentarily so popular »green chemistry« (Wang et al., 2000; Wang, 2002; Švancara et al., 2010).
Within the rapidly growing area of **non-mercury metallic electrodes**, the carbon paste as a special substrate offers a number of different variants, when one can choose amongst bismuth-film plated bare carbon pastes (BiF-CPEs), Bi(III)-solid compound containing carbon pastes (e.g., Bi_2O_3-CPE and BiF_3-CPE), or bismuth powder dispersed in bare carbon pastes (Bi-CPEs), including some of these variants being made from spedial carbon pastes of new generation, or being combined with the already modified carbon paste; e.g. BiF/Ze/CPE, where the modifier is zeolite (a natural clay bringing to the bare paste mixture better adhesive properties). All these configurations have certain advantages and drawbacks; nevertheless, their detailed characterisation is beyond the scope of this article and can be found elsewhere (*e.g*. Švancara et al., 2010).
In association with the previous survey of bismuth-versus-carbon-paste variants, it is interesting to notice that the spectrum of CP-based materials as the substrates for bismuth electrodes was relatively narrow being orientated on traditional (mineral and silicone oil-based) carbon paste mixtures. But the TCP-CPE, introduced above as the (bare) carbon paste electrode of choice for neonicotinoids is also applicable in the metallic film configuration. The first attempt of a kind has been made nearly twenty years ago (Švancara et al., 1993), when the TCP-CPE had been combined with a mercury film and - as the MF/TCP-CPE

configuration - successfully used in stripping voltammetry of some not readily reducible ions like Mn^{2+} or Zn^{2+}. Analogously, a coupling with a gold film; *i.e.*, the AuF/TCP-CPE configuration, had been tested as the working electrode for stripping voltammetry of As(III); but the ultimate choice being another gold plated-CPE (Chadim, 1999). When considering bismuth and its deposition onto the TCP-CPE, this combination has for the first time been examined for analysis of the neonicotinoids and therefore, it deserves a special attention being discussed below – in section **3**.

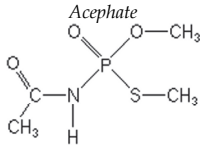
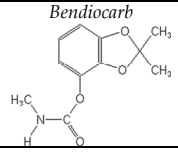
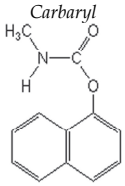
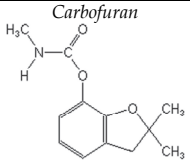
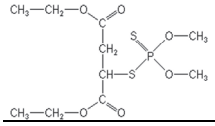
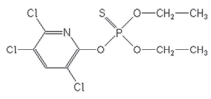
Carbon paste-based electrodes in retrospective reviews. The very first text of a kind on CPEs had been published by the inventor himself in the mid-1960s (Adams, 1963). Another related elaborate came a quarter of a century later; being focused predominantly on CMCPEs (Kalcher, 1990) and soon followed by two similar installments (Kalcher et al., 1995; Švancara et al., 2001a). In the recent years, the widespread area of carbon paste-based electrodes has been reviewed in a series of exclusive articles, including hitherto the most detailed chapter in an *Encyclopedia* (Kalcher et al., 2006) or the newest entry – the very first monograph on CPEs (Švancara et al., 2011). In all reviews cited, the reader can find almost complete information, with all newest achievements in the area which is further discussed in association with the key topic of this overview – with the electrochemistry and electroanalysis of neonicotinoid insecticides.

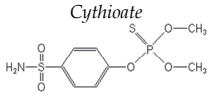
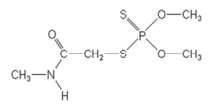
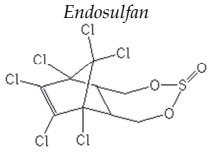
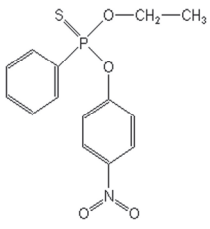
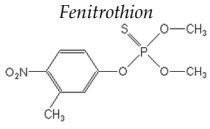
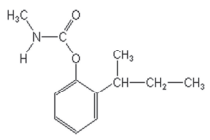
1.5 Carbon paste electrodes in analysis of insecticides

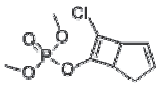
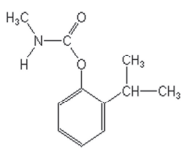
In contemporary literature (Kalcher et al., 2006; Zima et al., 2009a,b; Švancara et al., 2011), the individual methods for the determination of insecticides at CPEs, CMCPEs, and CP-biosensors have always been included within a group of the so-called **organic pollutants**; namely, together with other pesticides or animal poisons, with polyaromatic amines, nitro- and nitroso-derivatives, or various industrial products like dyes, catalysts, and food additives. The reason for such a grouping is that the respective electroanalytical procedures share usually some general features that correspond also to the main rules and recommendations for routine organic electrochemistry highlighted in the "fresh" review literature (see Zima et al., 2009a,b, Kalcher et al, 2009).

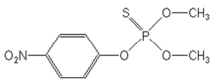
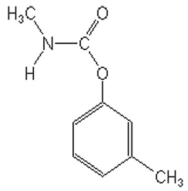
Regarding the insecticides as such, there is about three tens of different synthetic compounds that have already been analysed with the aid of carbon paste-based electrodes. Their (probably) complete survey is given in **Table 2**, specifying also the particular type of a CPE or a CMCPE, the technique of choice, a selection of typical experimental and instrumental parameters, as well as the basic electroanalytical characterisation via the linearity range or the limits of detection. Finally, the table also gathers the data on the individual samples, confirming that analyses of environmental specimens and commercial formulations had dominated. Herein, it should be quoted that the methods for the determination of the remaining pesticides (i.e., herbicides, fungicides, algaecides, rodenticides, molluscicides, acaricides, bactericides, and virucides) have also been reviewed and gathered in similar tables in two recent literature sources (Kalcher at al., 2006; Švancara et al., 2011)

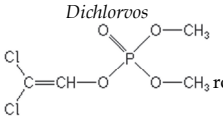
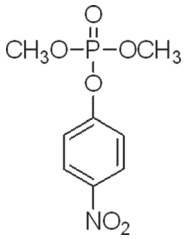
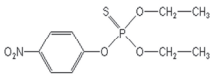
In the following sections, possibilities and limitations of **voltammetric measurements with carbon paste electrodes** are of interest, focused on the **neonicotinoid insecticides** and the authors' research with **tricresyl phosphate-based CPEs** used either in **the bare configuration** or as further chemically modified via electrolytic plating the electrode surface

Analyte (form / alter.n.)	Chem. specification	Type of CPE (modifier) [configuration]	Technique (mode)	Selected experimental conditions and analytical parameters; notes	Sample(s)	Ref (s)
Acephate 	methoxy-sulfanyl-phospho-acetamide	CNTPE (+ $Ru(bpy)_3^{2+}$) [EH-μD]	CE-ECL	EH: electrically heated electrochemilu-minescence detection; lin.r.: μM level; simult. detn. with *Dimethoate*	comm. forms	(Chen et al., 2007)
Bendiocarb 	dimethyl-benzo-di-oxo-ethylcarbamate	C/Nj (+ "C-18")	CV, DPV	modif.: ionex resin (50% in CP); o.c., MEx, BR-B (pH 5); LOD: 0.7 $\mu g \cdot mL^{-1}$	model solns., soils	(P. Hernandez et al., 1993)
Carbaryl 	1-naphthyl-methyl-carbamate	C/PO (+ polyamide) [ft-D]	CZE-AD	hydrolysis to phenolic ders. and detn.; simult. detn. with *Fenobucarb*, *Isopro-carb* & *Metolcarb;* LODs: $3\text{-}6\times10^{-8}$ M	environmental specimens	(Cheng et al., 2007)
Carbofuran 	subst. benzofuran methylcarbamate	C/PO (+ FePC) [CP-bio with enzymes]	AD (stat.)	inhibition effect of analyte; two diff. enzymes; LOD: 1×10^{-10} M;	model solns.	(Ciucu et al., 2003)
Carbophos 	di-MeO-phosphino-thio-butanoic acid e.	C/PO (Co^{II}-2,2'-dipy)	DPAdSV	studies on accum. mechanism, adsopt. pH-effect, interfs.; LOD: 1.2×10^{-9} M	model solns.	(Ulakhovicet al., 1998)
Chlorpyrifos 	diethyl-trichloro-py-phosphoro-thiolate	C/PO (+ *Zeolite Y*)	DPAdSV	study on ion-pairing accum. and effect of pH, modif.: nat. clay; lin.: 0.0001 - 2 ppm (t_{acc} = 80 s); interf. Studies	enviromental specimens	(Sirisha et al., 2007)
Clothianidin (see Table 1)	neonicotinoid	C/SO (unm.), C/TCP (unm.)	DPV (dir.)	s.e.: BR-B (pH 7.0), lin.r.: 2-25 $mg \cdot L^{-1}$	model solns.	(Papp et al., 2009a)

Analyte (form / alter.n.)	Chem. specification	Type of CPE (modifier) [configuration]	Technique (mode)	Selected experimental conditions and analytical parameters; notes	Sample(s)	Ref (s)
Cythioate 	organo-S-phosphate	C/PO (crown-ethers)	CV, DPV	studies on accum., re-oxidn. processes; lin.r.: 4×10^{-9}-9×10^{-8} M; RSD < 4%	model solns.	(Shaidarova et al., 1998)
Dimethoate 	dimethyl-amino-2-oxo-dithiophoshate	CNTPE (+ $Ru(bpy)_3^{2+}$) [EH-μD]	CE-ECL	EH: electrically heated electrochemilu-minescence detection; lin.r.: μM level; simult. detn. with *Acephate*	comm. forms.	(Chen, 2007)
Endosulfan 	subst. hexachloro-bis benzo-oxa-thiepine	C/PO (+ ionex "C-18") [trad. CPE + Cu^{2+}, in situ]	DPV	indirect detn. based on interaction of electroinactive EDS with Cu(II) / via the peak decrease; LOD: 40 ng·L^{-1}	model solns.	(El Bakouri et al., 2005)
EPN 	ethyl-O-nitro-Ph-Ph-phosphonothioate	a) CNTPEs (+ MeF, DNA) [μEs: needle type] b) C/PO (+ *Pseumonas putida* sp.+ OPH-enzyme)	a) ASV (trad. & *in vivo*) b) AD (stat.)	a) study with a series of w. electrodes, incl. GCE,AuE), MeF-: mercury film, 0.1M $NH_4H_2PO_4$; lin.r.: 10-210 ng·L^{-1}; b) CP-bio with modif. as bio-degrader, LOD: 1.6 ppb; detn. with *Fenitrothion*	a) orange, apple, and skin tissues b) lake water	a) (Ly et al., 2008) b) (Lei et al., 2007)
Fenitrothion 	diMeO-4-nitro-PhO -thioxo-phosphorane	C/PO (+ *Pseumonas sp.* + OPH-enzyme) [CP-bio + Co^{2+}, *in situ*]	AD (stat.)	modif: microbial cells (as biodegrader) 0.05M PhB (pH 7.5), E_w = 0.6 V / ref.; LOD: 1.4 ppb; simult. detn. with *EPN*	lake water	(Lei et al., 2007)
Fenobucarb 	2-butan-yl-2-phenyl methyl-carbamate	C/PO (+ polyamide) [ft-D]	CZE-AD	hydrolysis to phenolic ders. and detn.; simult.detn. with *Carbaryl*, *Isoprocarb* and *Metolcarb;* LODs: 3-6 × 10^{-8} M	environmental specimens	(Cheng, 2007)

Analyte (form / alter.n.)	**Chem. specification**	**Type of CPE (modifier) [configuration]**	**Technique (mode)**	**Selected experimental conditions and analytical parameters; notes**	**Sample(s)**	**Ref (s)**
Hostaquick 	chloro-bicyclo-di-methyl phosphate	C/Nj + m. (enzyme + Co-PC)	AD, HA	detn. based on analyte inhibition / bio-enzymatic effect; PC: phthalocyanine LOD: 3×10^{-4}- 0.1 g·L^{-1}	model solns.	(Skladal, 1991)
Imidacloprid (see Table 1)	neonicotinoid	a) C/SO, C/nTD, C/TCP b) C/SO, C/TCP [all CPEs as unm.] c) Bi powder bulk modified C/TCP	a,b,c) DPV (dir.)	a) comp. of three diff. CPEs (made of SO, tetradecane & tricresyl phosphate) s.e.: BR-B (pH 7.0), lin.r.: 1.7-30 mg L^{-1} b) comp. of two diff. CPEs (made of SO & tricresyl phosphate) s.e.: BR-B (pH 7.0), lin.r.: 2-25 mg L^{-1} c) Bi powder bulk modified CPE based on tricresyl phosphate, s.e.: BR-B (pH 7.0), lin.r.: 1.6-47.6 mg L^{-1}	a) river water, comm. preps. b) model solns.	a) (Papp et al., 2009, b) b) (Papp et al., 2009, a) c) Guzsvány et al., 2011)
Isoprocarb 	iso-propan-2-phenyl methyl-carbamate	C/PO (+ polyamide) [ft-D]	CZE-AD	hydrolysis to phenolic ders. and detn.; simult. detn. with *Carbaryl, Fenobu-carb, Metolcarb*; LODs: 3-6 × 10^{-8} M	environmental specimens	(Cheng, 2007)

Analyte (form / alter.n.)	Chem. specification	Type of CPE (modifier) [configuration]	Technique (mode)	Selected experimental conditions and analytical parameters; notes	Sample(s)	Ref (s)
Methyl parathion 	(subst. p-nitro-phen-oxy-organophosphate	a) C/PO (+ FePC) [CP-bio with enzymes] b) C/Nj (+ "C-18") c) C/MO (+ enzyme) d) CNT-IL ($BMIMPF_6$) [CP-film at the GCE] e) C/PO (MBD + OPH) f) C/PO (unm.) g) C/PO (+ mep-ZrO_2) h) C/PO (+ nano-ZrO_2)	a) AD (stat.) b,d) DPV c) FIA-EC e) LSV, AD (stat.) f) SWAdSV g) DPASV h) SWASV	a) nal. inhibition / bioenzymatic effect; b) interfs. studies; LOD: 7.9 ng mL^{-1}; c) catalysed hydrolysis; 0.2-1.0 µM; d) catalytic effect of both CNTs & IL, o.c. accum., PhB (pH 7), LOD: 1 nM e) modif: microbial cells (degrader) + enzyme; LODs: 0.3-5.3 ppb, simult. detn. with *Paraoxon* and *Parathion;* f) accum by adsorpt., LOD: 0.05 µM; g,h) o.c. accum. with strong affinity of P=O to mdf., DLs: a) 5 nM, b) 5 μgL^{-1}	a) model solns. b) lake water c) well water (spiked) d) nat. water, soil e) soil extracts f) model mixts. g) apple tissue h) diff. water specimens	a) (Skladal, 1991) b) (L. Hernandez et al., 1993) c) (Mulchandani et al., 2001, a) (Mulchandani et al., 2001, b) d) (Fan et al., 2008) e) (Lei et al., 2004; Lei et al., 2005) f) (Liu & Lin, 2005) g) (Tan et al., 2010) h) (Parham & Rahbar, 2010)
Metolcarb 	methyl-hydroxy-2-Ph-methylcarbamate	C/PO (+ polyamide) [ft-D]	CZE-AD	hydrolysis to phenolic ders. and detn.; simult.detn. with *Carbaryl, Fenobu-carb, Isoprocarb* LODs: 3-6 $\times 10^{-8}$ M	environmental specimens	(Cheng, 2007)
Nitenpyram (see Table 1)	neonicotinoid	C/SO, C/TCP [both CPEs as unm.]	DPV (dir.)	s.e.: BR-B (pH 7.0), lin.r.: 2-25 mg L^{-1}; detn.with *Clothianidin* & *Imidacloprid* electrolytic activation of CPEs tested	model solns.	(Papp et al., 2009a)

Analyte (form / alter.n.)	Chem. specification	Type of CPE (modifier) [configuration]	Technique (mode)	Selected experimental conditions and analytical parameters; notes	Sample(s)	Ref (s)
Dichlorvos 	dimethyl-2,2-dichlo-rovinyl phosphate	C/PO (+ Co-PC) [CP-bio with enzymes]	AD (stat.)	detn. based on analyte inhibition / bio-enzymatic effect; PC: phthalocyanine, LOD: 2×10^{-4}- 0.1 $g\cdot L^{-1}$; RSD < ±3 %	model solns.	(Skladal, 1991)
Paraoxon-methyl 	subst. p-nitro-phen-oxy-organophoshate	a) C/PO (+ OPH-enzyme) b) C/PO (FePC + m-ezs) c) C/PO [+ *Arthrobacter* sp. + OPH-enzyme (m)] d) C/PO [+ *Pseudomonas putida JS444* sp + OPH]	a) FIA-EC b) AD (stat.) c) AD (stat.) d) LSV	OPH: phosphorus-hydroxylase; add. membrane + 2 enzymes; a,b) PhB, (pH 7-8), c) PhB + Na-citrate (pH 7.5) LODs: a) 0.1 µM, b) 1×10^{-10} M, c) 2.8 ppb; c) detn. with *Methylparathion* d) mdf: microbial degrader + enzyme, detn. with *Parathion, Methylparathion*	a) well water (spiked) b) model solns. c) soil extracts d) model solns.	a) (Mulchandani et al., 2001, a) (Mulchandani et al., 2001, b) b) (Ciucu, 2003) c) (Lei et al., 2004) d) (Lei et al., 2005)
Parathion 	subst. p-nitro-phen-oxy-organophoshate	a) C/PO [+ *Pseudomonas putida JS444* sp + OPH] b) C/PO (+ MIP)	a) LSV b) CV, DPV	a) mdf: microbial degrader + enzyme; detn. with *Paraoxon, Methylparathion* b) mdf.: molecularly imprinted polymer (sel. recogn. / extr. effect), l: 2-900 nM.	a) model solns. b) ground water, vegetables	a) (Lei et al., 2005) b) (Alizadeh, 2009)

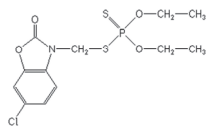
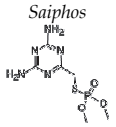
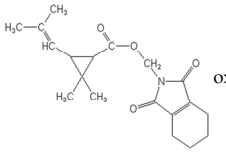
Analyte (form / alter.n.)	Chem. specification	Type of CPE (modifier) [configuration]	Technique (mode)	Selected experimental conditions and analytical parameters; notes	Sample(s)	Ref (s)
Phosalon 	chloro-diethoxy-thio phosphin-benzoxazol	C/PO (+ Co^{II}-2,2'-dipy)	DPAdSV	studies on pH-effect, adsorpt. & accum mechanism, interfs.; LOD: 5.5×10^{-9} M	model solns.	(Ulakhovic, 1998)
Saiphos 	(subst. diamino-phos-phino-thioyl-triazine	C/PO (+ crown-ethers)	CV, DPV	studies on accum. & reoxidn. process; lin.r.: 5×10^{-9}-1×10^{-7} M	model solns.	(Shaidarova et al., 1998)
Tetramethrin 	subst. cyano-phen-oxy-benzylalcohol	C/PO (+ *Sepiolite*)	DPV	studies on pH, accum. process, interfs. o.c., MEx, pH 5.3 + 12, LoD: 45 µg L^{-1}	water samples, soils	(Hernandez, 1989)
Thiamethoxam (see Table 1)	neonicotinoid	C/TCP (unm.)	DPV (dir.)	detn. without accum., s.e.: BRB (pH 7) lin.r.: a) 3.7-41.5 µg·mL^{-1}, RSD = ± 1.3%	river water, comm. prep. (*Actara 25-WG*)	(Papp et al., 2010)

Table 2. Determination of insecticides at carbon paste-based electrodes, sensors, and detectors. Survey of methods and selected studies.

with a relatively compact structure of metallic bismuth; i.e., as the **Bi(F)-TCP-CPE** or **bulk modified, $Bi_{5\%}$-TCP-CPE**, detection systems. By discussion the results and observations from the previous investigations, it is shown that all types of the TCP-CPE may offer a very fine electroanalytical performance in analysis of typical representatives of the neonicotinoid insecticides.

2. Choice and characterization of carbon paste electrodes for analysis of the neonicotinoids

2.1 Voltammetric behaviour of the neonicotinoids at CPEs and the effect of pasting liquid

As found out (Guzsvány et al., 2005; Guzsvány, 2006; Gaál et al., 2007; Papp et al., 2009b; Papp et al., 2010; Papp et al., 2011; Guzsvány et al., 2011), the voltammetric analysis of nitroguanidine and nitrometylene neonicotinoids at carbon-based working electrode revealed only the first reduction step, giving rise to the single analytical signal, which is similar to the previous experiments with the mercury electrode; see scheme (**1**) above.

Typical behaviour of the neonicotinoids at carbon paste-based electrodes is then depicted in **Fig. 1**, illustrating the initial testing (Papp et al., 2009b) in the same solution of *Imidacloprid* by using different types of CPEs, but with the same surface diameter of 2 mm. The respective CPEs had been prepared from three different liquid binders: **1**) silicone oil (the electrode denoted as "SO-CPE"), **2**) n-tetradecane ("C14-CPE"), and **3**) tricresyl phosphate ("TCP-CPE"). Moreover, in order to compare the individual reduction signals with traditional type of carbon electrode, identical voltammetric measurements were made also with **4**) the glassy carbon electrode (GCE, with the same diameter).

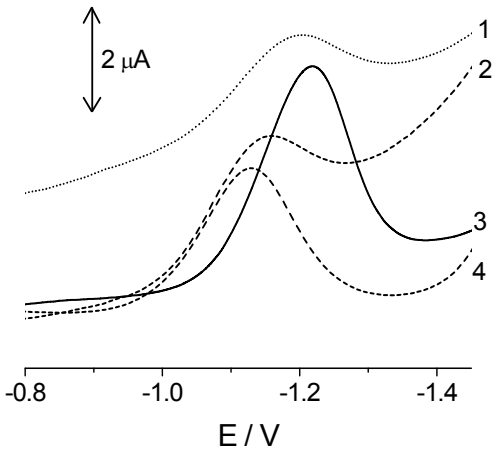


Fig. 1. Comparison of the DPV signals obtained at different working electrodes: SO-CPE (1), C14-CPE (2), TCP-CPE (3) and GCE (4) in the same solution of *Imidacloprid*.
Experimental parameters: 25 mV s^{-1}, pulse amplitude 50 mV, pulse width 50 ms, c= 33.3 μg mL^{-1}, pH 7.0.

In all cases, one reduction peak was observed, but the reduction signals are different. The voltammogram recorded at the TCP-CPE (curve 3) is best developed, with most satisfactory signal-to-noise characteristics compared to the remaining signals at SO-CPE, C14-CPE, as well as GCE (curves 1, 2, and 4). As discussed in the original papers such behavior of the TCP-CPE was likely due to its polarity (Švancara and Vytřas, 1993; Papp et al., 2009b), facilitating the contact of polar neonicotinoid molecules with the electrode surface. Also, it can be noticed that the maximum of reduction peak of model compound is of about 0.1 V more negative at the TCP-CPE compared to both C14-CPE and the GCE. In conclusion, it was demonstrated that among the investigated binding liquids (i.e., silicone oil, *n*-tetradecane and tricresyl phosphate (TCP), the latter had offered the best electroanalytical performance for analytical applications (Papp et al., 2009b) and, naturally, the TCP-CPE is of continuing interest in our further investigations.

Thus, the same electrode has also undergone a special characterization by means of microscopic imaging using scanning electron microscopy (SEM). More specifically, the graphite powder (type "CR5"), one of the two basic component of the electrode, and the resultant TCP-CPE were observed and their microstructure is shown in **Fig. 2**. As reported earlier (Švancara and Vytřas, 1993 and Papp et al., 2009b), two different morfologies of the SEM sample can be observed. Characteristic particles of CR5 graphite with distinct sharp

edges are well recognizable on the carbon powder surface (A), while in the case of the TCP-based paste, the surface looks notably smoother and more uniform. At some images (e.g. those reported in Švancara et al., 1996; Papp et al., 2009b; Papp et al., 2011), this texture had moreover manifested a notable tendency to form very compact aggregates, making thus the TCP-paste quite unique in comparison with the other common mixtures. Although the exact reflection of this feature into the behaviour of the TCP-CPE has not been yet fully explained, this unique microstructure is undoubtedly behind some specifics of this unusual CPE (Švancara et al., 2011).

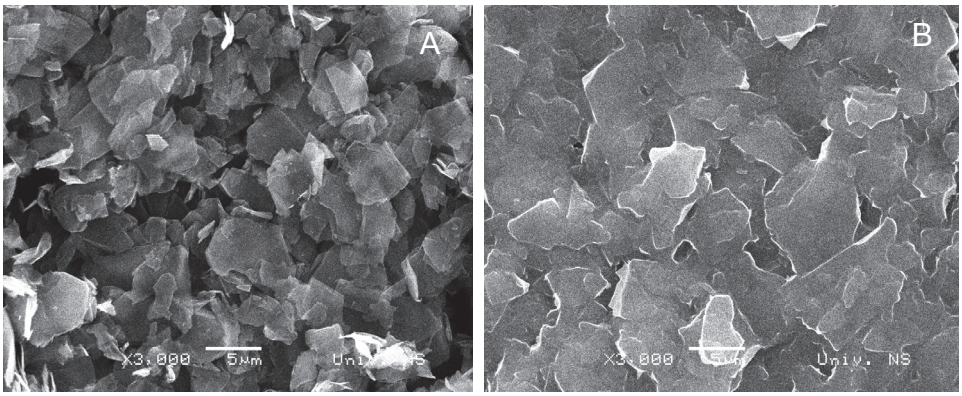


Fig. 2. SEM image of the CR5 graphite powder (A) and TCP-CPE (B).

Herein, one can quote two most distinct abnormalities of the TCP-CPE compared to common CPEs, as well as other carbonaceous electrodes. At first, it is unusually high signal-to-noise ratio and its overall magnitude, when - in some cases - the respective values may reach even a ten-fold intensity of that being typical for Nujol oil (Švancara et al., 2002) - or silicone oil-based CPEs (Švancara et al., 2002.; Papp et al., 2009b). Secondly, it is a shorter lifetime of the TCP-CPE given by higher volatility of tricresyl phoshate compared to traditional oily binders (Švancara and Vytřas, 1993; Papp, 2011; Papp et al. 2011). According to purely empirical observations, an average lifetime of a TCP-CPE is about two weeks only; afterwards, the paste becomes desiccated and unavoidably disintegrates, losing quickly all typical properties. This can be clearly documented in **Fig. 3**, visualising the recent experiment on the ageing of the TCP-CPE. As can be seen, the "old" paste has lost totally its sensitivity to the model analyte, exhibiting also notably higher background. (Both phenomena indicate the loss of the binder, as well as the increased content of graphite in the desiccated mixture.) Newly (Papp et al., 2011), it was shown that this drawback of the TCP-CPE can be minimised by enveloping the tip of the electrode with Parafilm® and storage in the fridge (at ca. 4 ˚C) - in other words, by preventing the evaporation of tricresyl phoshate - and the overall lifetime can thus be extended up to several months of use (!)

2.2 Development and optimisation of the method for the determination of neonicotinoids at the bare TCP-CPE

Our previous investigations with the bare TCP-CPE gathered in the respective reports (Papp et al., 2009a, 2009b, 2011) have finally resulted in a simple procedure, allowing us also quantitative analysis of selected neonicotinoids, as well as sensitive monitoring during their

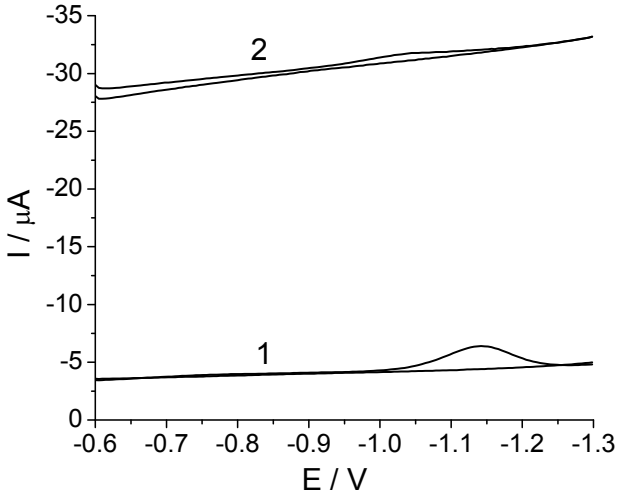


Fig. 3. Comparison of DPV signals obtained for Imidacloprid with the TCP-CPE: 1) one day after preparation, 2) ten days after preparation in solution with the same concentration of model substance (c = 33.3 μg mL^{-1}). Note: below are shown the corresponding baselines (blank solution).

degradibility in the course of time. All the principal features of these measurements are briefly summarised in the following sections.

In the first phase, elaboration of the proper procedure (Papp et al., 2009a, 2009b, 2010) required to investigate / optimise several key parameters; namely, the effect of dissolved oxygen (see **Fig. 4**), electrode conditioning (**Fig. 5**), acidity of the supporting electrolyte (not shown), or accumulation capabilities of model neonicotinoids (**Fig. 6**); again, when keeping in mind specific signal-to-noise characteristics of the TCP-CPE.

Effect of Oxygen and Electrode Conditiong. It was shown that the purging of the sample solutions with inert gas (**Fig. 4**, curve 2) was necessary to suppress the effect of the dissolved oxygen (curve 1). In the case of the neonicotionoids, this step had been found inevitable despite the previous observation that the TCP-CPE would have exhibited generally lowered signal of oxygen reduction (Švancara et al., 1993; Kalcher et al., 2006). Further, as mentioned earlier (Papp et al., 2009b) and shown in Fig. 5, electrochemical conditioning of the TCP-CPE by potential cycling was found beneficial in the potential range of interest, resulting in effective lowering of the background and more stable voltammetric signals.

Accumulation Study. A possible adsorptibility of the neonicotinoids as a way of potentially effective pre-concentration at the TCP-CPE surface was tested with *Imidacloprid*, when setting the respective time periods within an interval of 1-20 min. The whole experiment is given in Fig. 6, where curve 1 represents the initial stage, *i.e.,* the DP-voltammogram No. 1 (obtained by reducing the model substance). Afterwards, the electrode was left in the same solution (with pH 8.0) for 20 min without potential applied; the subsequent DPV record being No. 2. As seen, there is almost no difference between both records,

suggesting us that *Imidacloprid* have had no tendencies to be adsorbed onto the TCP-CPE surface.

Reproducibility Studies. At the TCP-CPE, the signals for the four investigated compounds were checked as the corresponding replicates for a model concentration of each neonicotinoid. This assay had been included to reveal again whether or not the system studied would exhibit some accumulation capabilities, such as sorption affinity. **Fig. 7** showing a nonet of replicates confirming good reproducibility for a signal of 3.72 μg mL^{-1} *Thiamethoxam*, within the time interval of approx. 30 min, with no evidence for a spontaneous accumulation and with the RSD being less than 2.5% rel. Nearly the same results has then been obtained also for the remaining three neonicotinoids (Papp, 2011).

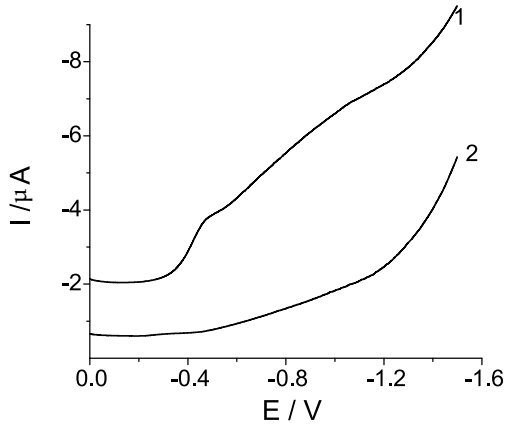


Fig. 4. Effect of presence (1) and absence (2) of dissolved oxygen on the baseline of DPV signals at pH 7.0 (after Papp et al., 2009a).

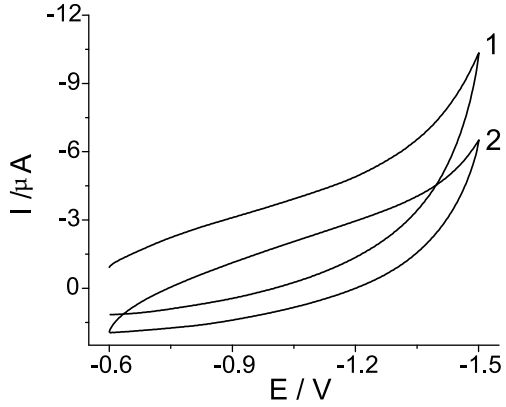


Fig. 5. Potential cycling of TCP-CPE. Exp. parameters: pH 7.0; v = 100 mV/s^{-1} (1: 1st cycle; 2: 10th cycle) (after Papp et al., 20011).

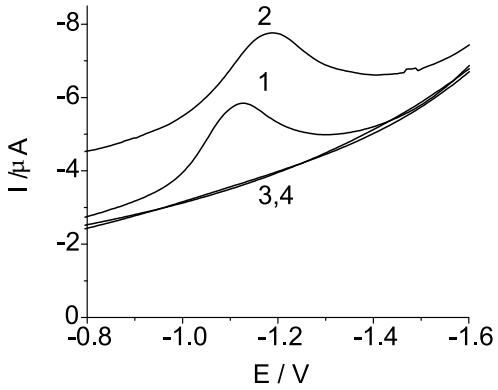


Fig. 6. An assay on potentially adsorptive behavior of *Imidacloprid* at the TCP-CPE: 1) DPV reduction, 2) DPV reduction after 20 min. of accumulating under open-circuit conditions, and 3,4) base-lines before and after 1st measurement. Other experimental parameters: pH 8.0, c(IMI) = 33.3 μg mL^{-1}; scan rate, 25 mV s^{-1}, pulse amplitude, +50 mV, pulse width 50 ms

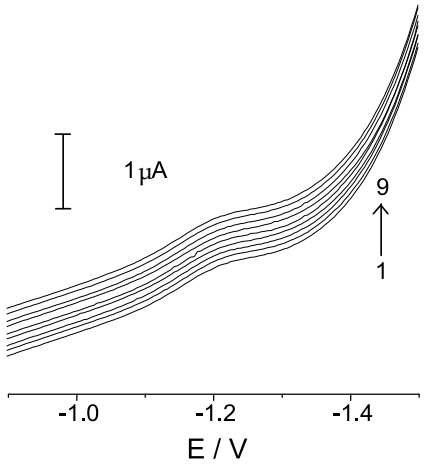


Fig. 7. The reproducibility of the analytical signal for *Thiamethoxam* at the TCP-CPE. Model concentration, c(TH) = 3.7 μg mL^{-1} measured in an time interval of 30 min (Papp, 2011).

Comparison of two different TCP-CPEs. After the checking the reproducibility of the analytical signals and their stability in time, the next step was to compare the electroanalytical performance of two individual TCP-CPEs in analysis of the same sample solution. This assay was assembled for an eventuality if a series of identical working electrodes would be needed for routine analysis; for example, in the field monitoring at different locations. For this purpose, *Clothianidin* was selected as the substance of choice being determined at two identical TCP-CPEs that had been prepared from the same batch of carbon paste (see **Fig. 8**). In both cases, the electrodes tested showed almost the same performance, confirming the applicability of the TCP-CPE to the decentralized determination; i.e., at more sites in one time. Herein, however, it should be emphasised that any joint (absolute) calibration is not

feasible and each electrode has to be prepared for quantitative analysis individually. (This is given by the specific character of the carbon paste electrode material, when every respective electrode under actual use represents a unique item with its own characteristics – for other details, see Švancara and Schachl, 1999).

Other practical notes and hints. The ultimate method for the determination of *Imidacloprid* and *Thiamethoxam* at the TCP-CPE in model solutions, as well as real samples (various commercial formulations), has already been elaborated into detail a couple of years ago (Papp et al., 2009a; 2009b; 2010), including the assesment of the individual instrumental options and experimental parameters for the determination of *Clotianidin* and *Nitenpyram*. In brief, both neonicotninoids of interest could be determined at the low ppm level from 1 or 2 μg mL^{-1}, respectively, up to 35 and 45 μg mL^{-1} with the RSD lower than 2.4% rel.

As discussed therein, the voltammetric characteristics of the two compounds had depended strongly upon the pH of the sample solution; for each the optimum being at pH 7.0. Finally, limits of quantitation (LOQs) estimated in a conventional way (3s) were found to be at the low μg mL^{-1} level for all the neonicotinoids included in the study – i.e., for *Imidacloprid, Thiamethoxam, Clothianidin*, and *Nitenpyram*, too.

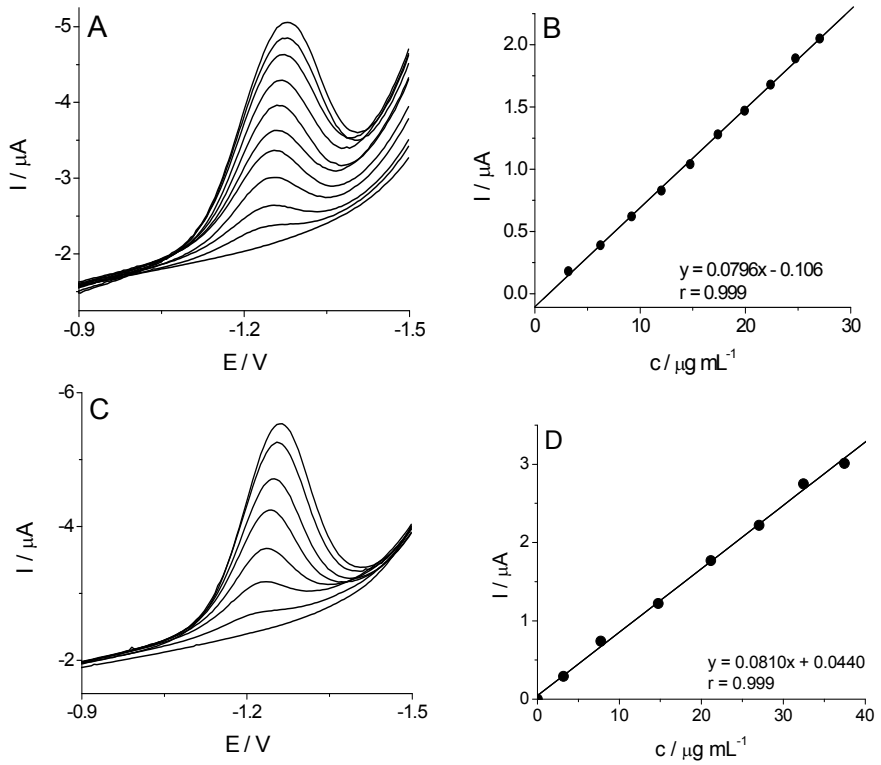


Fig. 8. Differential pulse voltammograms of clothianidin recorded at different concentrations of two TCP-CPE identical composition. A) TCP-CPE-1 and C) TCP-CPE-2; B) and D) the corresponding calibration curves (Papp, 2011).

Concerning the real analysis, the method for the determination of neonicotinoids at the TCP-CPE (Papp et al., 2009a; Papp et al., 2009b; Papp et al., 2010; Papp et al., 2011; Papp, 2011) in combination with DPV was applied to identify and quantify *Imidacloprid* and *Thiamethoxam* in samples of river water and some commercial formulations; namely: "Macho 200 SL" (for the respective analysis, **Fig. 9**), "Confidor 200 SL" and "Actara 25 WG"), when using the standard addition method with multiple injection of the standard (in aliquots).
It can be stated that all the tests performed had shown a very good correlation between the determined and the nominal (added) amounts of each. And, in all cases, the results of electrochemical analyses had been compared to the reference method employing HPLC/DAD, showing good agreement for each sample.
Finally, the TCP-CPE was also examined in the monitoring of photolytic and photocatalytic degradation of *Thiamethoxam* (for the latter, with TiO_2 particles as the photocatalyst, Degussa 25) under natural insolation (see **Fig. 10**), as well as in the dark regime (Fig. 11, lines 3, 4). It was shown that, under insolation in the autumn period (September, 2009), the photolytic processes followed the first-order reaction kinetics, while the photocatalytic process followed a kinetics of the pseudo-first order. As the DPV method and the reference HPLC/DAD had given very similar kinetic data, the voltammetric method with TCP-CPE could thus be evaluated as fully applicable to obtain the fast (orientation) information about the concentration of the neonicotinoids during their photodegradation (Papp, 2011).

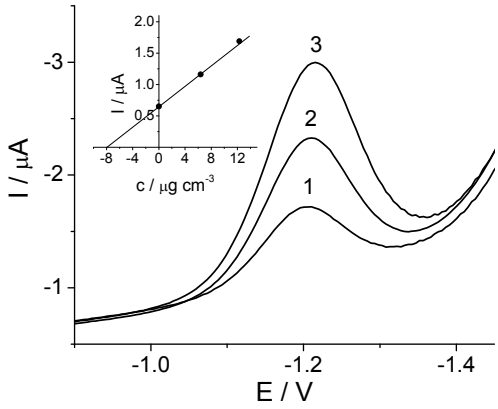


Fig. 9. Determination of *Imidacloprid* in commercial formulation "Macho 200 SL". 1) sample and 2,3) two successive additions of standard solutions of *Imidacloprid*.

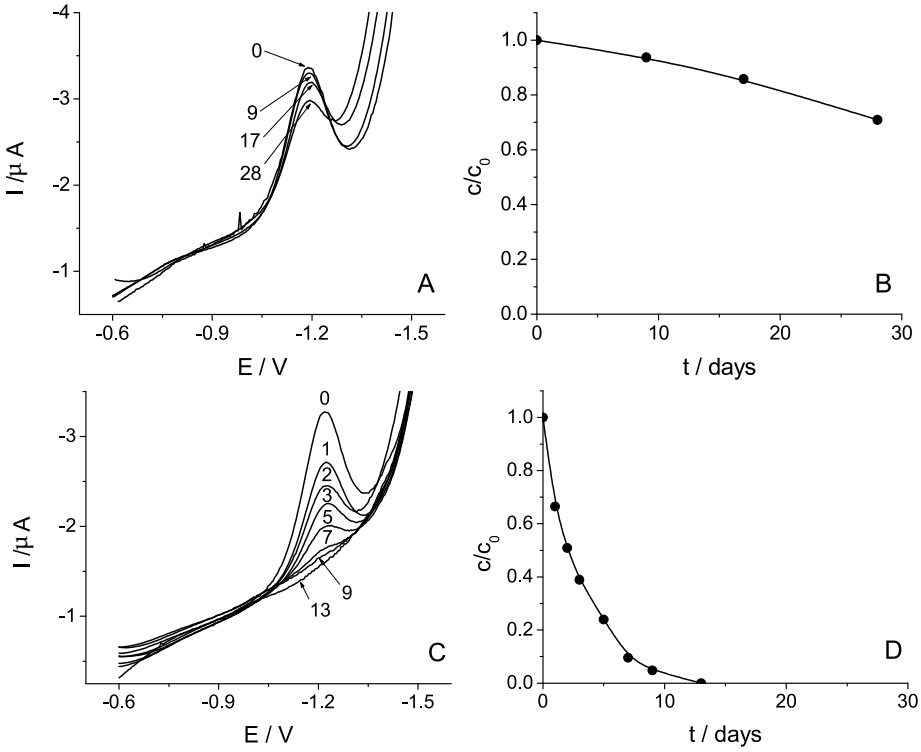


Fig. 10. Differential pulse voltammograms recorded with TCP-CPE during photolytic (A) and photocatalytic (C) degradation of *Thiamethoxam* solution under natural insolation, as well as the corresponding kinetic curves (B and D). The initial concentration of the insecticide was 116.7 μg mL^{-1}. The sample aliquots were diluted with adequate Britton – Robinson buffer solutions pH 7.0. Numbers on DPV curves indicate the days of sampling (Papp, 2011).

In contrast to the results obtained under natural insolation, the dark-regime measurements with *Thiamethoxam* had not reveated any significant changes in the presence and absence of the photocatalyst as can be seen in **Fig. 11** overleaf. Under natural insolation promoted by the presence of TiO_2, the half-life time of the nitroguanidine electroactive site in the *Thiametoxam* molecule had been ascertained to be about just 2 days. The fast removal of *Thiamethoxam* in this case could be attributed to the presence and specific functioning of the rutile form of TiO_2 (Abramović et al. 2007; Guzsvány et al., 2010).

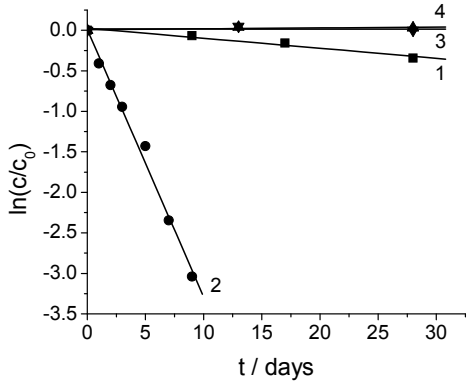


Fig. 11. Comparison of the kinetics of the photolytic (1) and photocatalytic (2) degradation of *Thiamethoxam* monitored by DPV with TCP-CPE. The lines for the monitoring of the stability of *Thiamethoxam* in dark in absence (3) and presence (4) of TiO_2 are also presented. The initial concentration of the insecticide was 116.7 μg mL^{-1}.

In general, the developed procedure could then be recommended as a convenient tool for the determination of *Thiamethoxam* during the photodegradation without a sample clean-up, which may further lower overall expenses. Though the chromatographic technique has been found to provide more detailed information about the system monitored, voltammetry with TCP-CPE that utilises fine electroactivity of all typical neonicotinoids represent, in our opinion, an attractive and low-cost alternative for environmental screening, being also capable of gaining information about the actual pollution with the insecticide and its eventual concentration changes during the photolytic and photocatalytic degradation.

3. Bismuth modified TCP-CPEs in analysis of selected neonicotinoids

3.1 Bismuth-film plated TCP-CPE for the determination of *Imidacloprid*

As mentioned above, the bismuth-film plated tricresyl phosphate-based carbon paste electrode; i.e., the BiF/TCP-CPE configuration has firstly been tested and used for the determination of some neonicotinoids. Similarly like other bismuth-film plated glassy carbon electrodes (Hutton et al., 2004; Guzsvány, 2006; Guzsvány et al., 2006c; Gaál et al., 2007; Guzsvány et al., 2008b; Claux and Vittori, 2007; Moreno et al., 2009; Nigović et al., 2009; Li et al., 2010; de Figueiredo-Filho et al., 2010 and the references herein), also this variant has undergone the thorough characterization. The individual studies included also the comparison of TCP-CPE with Nujol oil and silicone oil-containing carbon pastes ("Nj-CPE" and "SO-CPE", respectively) in their electroanalytical performance for model solutions of *Imidacloprid*. In the case of the TCP-CPE and SO-CPE, the plating was carried out *ex situ* in (quiet) solution consisting of 0.02 M $Bi(NO_3)_3$, 1 M HCl, and 0.5 M KBr in an analogy to the previous procedure for the bismuth deposition onto the glassy carbon substrate electrode - GCE (Guzsvány et al., 2006c; Gaál et al., 2007; Guzsvány et al., 2008b). The choice of the deposition potential within this study for the respective bismuth-film

electrodes is illustrated in **Fig. 12** (A and B): the maximum of the bismuth reduction peak is the most convenient plating potential. On the other hand, images "C" and "D" demonstrate the differences in sensitivity for both BiF/TCP-CPE and BiF/SO-CPE with respect to *Imidacloprid*. In contrast to the sharp reduction peak obtained at the BiF/TCP-CPE, the reduction signal obtained at both bare TCP-CPE and SO-CPE are much lesser, which is also the case - rather surprisingly - of the BiF/SO-CPE responding with ca. 5x less intensity compared to the TCP counterpart. Furthermore, in contrast to measurements with the bare TCP-CPE that need to remove dissolved oxygen (see par. **2.1**), the presence of bismuth film at the surface had allowed to run the voltammetric scans even in the presence of oxygen, which is widely appreciable benefit of using bismuth-film based electrodes in cathodic measurements (Wang, 2005).

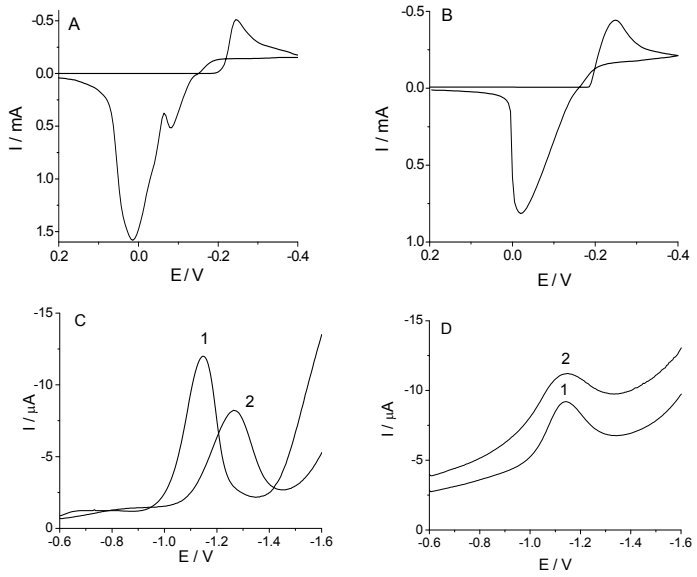


Fig. 12. Cyclic voltammogram recorded with two types of TCP in the solution consisting of 0.02 M $Bi(NO_3)_3$, 1 M HCl and 0.5 M KBr, (v =25 mV s^{-1}): A) TCP-CPE and B) SO-CPE and comparison of DPV signals obtained at two different bare and surface modified substrate working electrodes: C) BiF/TCP-CPE (1) and TCP-CPE (2) and D) BiF/SO-CPE (1) and SO-CPE (2) in the same *Imidacloprid* solution while c= 33.34 μg mL^{-1} and pH 7.0.

3.2 Bismuth bulk-modified TCP-CPE for analysis of *Imidacloprid* and *Nitenpyram*

The tricresyl phosphate-based CPE in the role of substrate for bismuth has also been tested in the alternative configuration – with a fine bismuth powder mechanically embedded and thus dispersed into the TCP-CP mixture. This "pseudo"-film arrangement functioning almost identically like common bismuth-film configurations (Hočevar et al., 2005; Švancara et al., 2006) seemed to be particularly convenient in combination with polar molecules of TCP, allowing us to accomplish the sensitive determination of selected neonicotinoid insecticides

(Papp et al., 2009a; Papp et al., 2009b; Papp et al., 2010; Papp et al., 2011) if the powdered bismuth is incorporated into the TCP-CPE at the appropriate amount (Guzsvány et al., 2011). The scanning electron microscopic (SEM) imaging of the surface microstructure of the TCP-CPE containing 5% (w/w) bismuth ("$Bi_{5\%}$/TCP-CPE") has shown that the binary carbon paste matrix contains a rather homogenous solid phase with randomly distributed irregularities that are, in fact, the aggregates of tiny Bi particles (see **Fig. 13**). This was confirmed by energy dispersive spectrometric measurements (EDS; **Fig. 14**) performed on two different sites of the actual $Bi_{5\%}$/TCP-CPE surface, selected so that both of them contains the mentioned Bi-aggregates dispersed in the TCP-CPE matrix (Guzsvány et al., 2011).

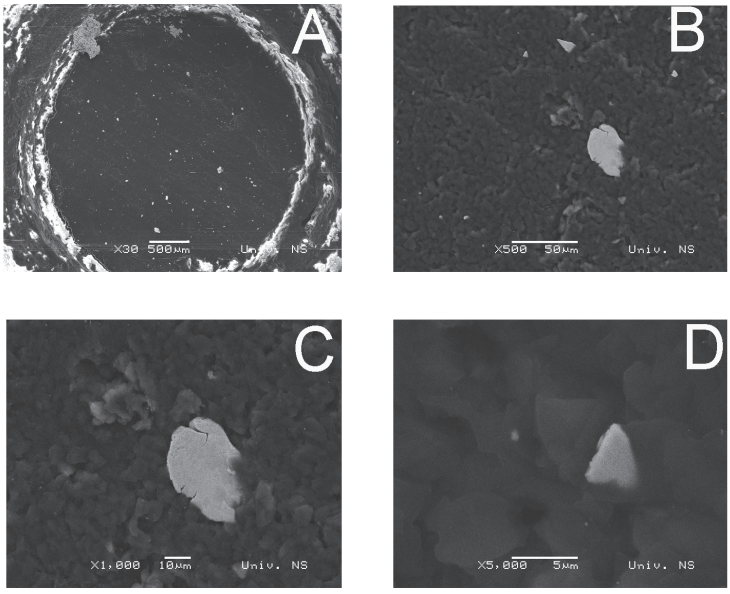


Fig. 13. Scanning electron micrographs of $Bi_{5\%}$/TCP-CPE obtained at different magnification.

The applicability of the bare (unmodified) TCP-CPE and of the $Bi_{5\%}$/TCP-CPE as two different working electrodes to determine the model compound, *Imidacloprid*, has been examined by comparing at the same experimental conditions (Guzsvány et al., 2011). In the case of both electrodes, the reduction peak was observed at ca. −1.2 V vs. ref., but the reduction signals were somewhat different. In comparison with TCP-CPE, a potential shift of about 50 mV towards the less negative potentials could be observed for the Bi5%/TCP-CPE, resulting in better insensitivity of this electrode to the presence of oxygen so that there was no need to purge the solution with inert gas (Guzsvány et al., 2011). Similar observations with both TCP-CPE and $Bi_{5\%}$/TCP-CPE have also been made for analysis of *Nitenpyram* as documented in **Fig 15**. The whole study also permitted to estimate the limit of quantification (LOQ); the respective values being down to 1.9 µg mL^{-1} and 1.6 µg mL^{-1} *Nitenpyram* for TCP-CPE and $Bi_{5\%}$-TCP-CPE, respectively. Also, the reproducibility studies swith both electrodes were performed, when six replicates with each and in the model solution of 15.4 µg mL^{-1} *Nitenpyram* gave the RSD typically less than ±2.5%

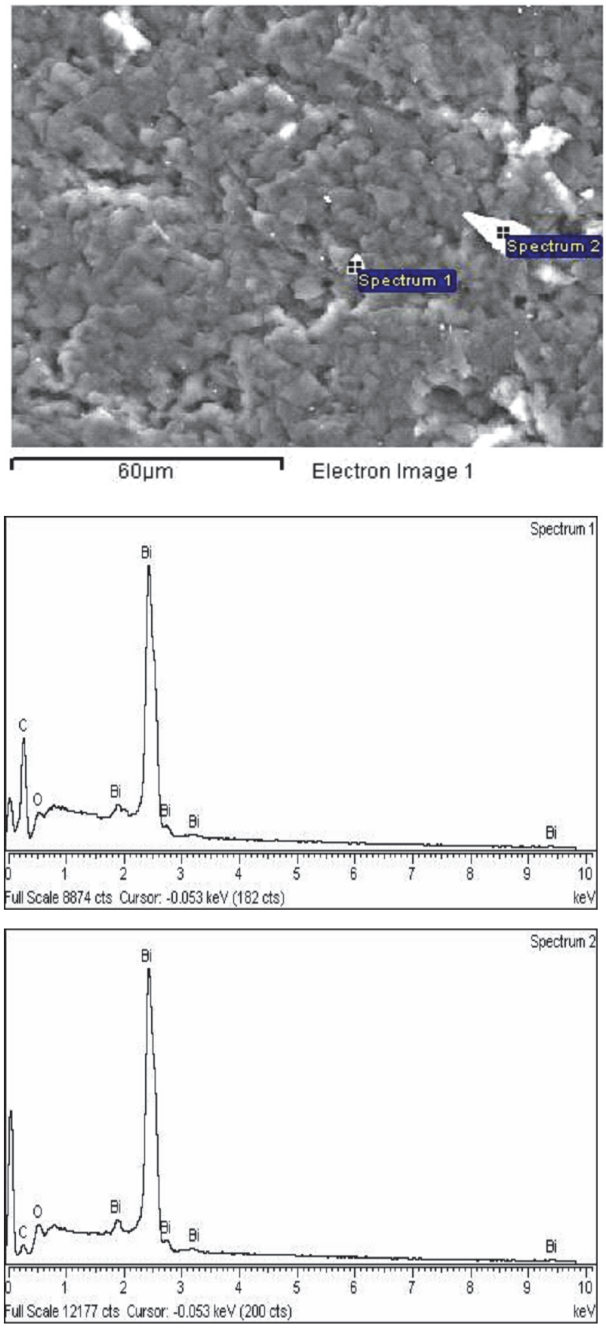


Fig. 14. Representative part of the $Bi_{5\%}$/TCP-CPE surface and EDS microanalysis spectra of the bismuth particles inside the actual surface area.

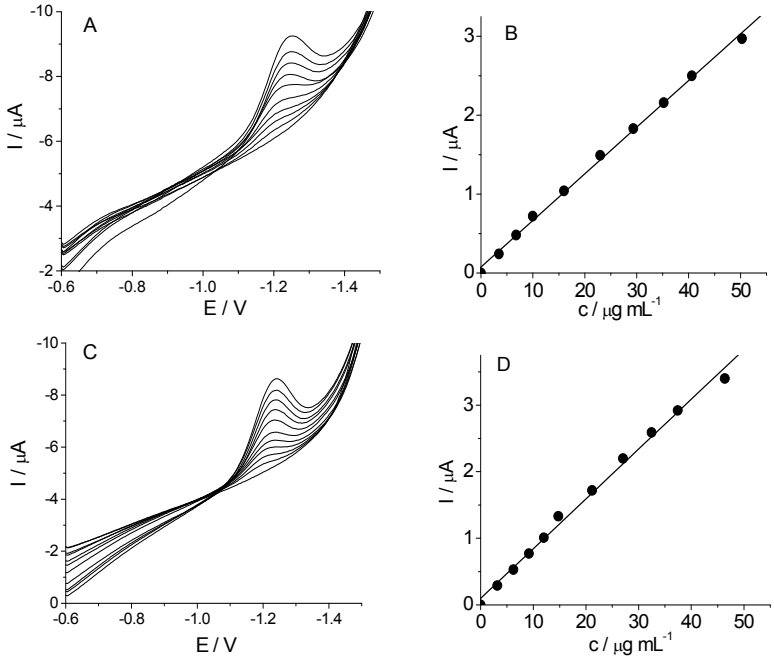


Fig. 15. Differential pulse voltammograms of *Nitenpyram* recorded at different concentrations of the compound at $Bi_{5\%}$/TCP-CPE (**A**) and TCP-CPE (**C**) with the corresponding calibration curves (**B** and **D**).

All these results have shown that there were no significant differences in the overall electroanalytical performance in direct cathodic determinations and, in fact, both TCP-CPE and $Bi_{5\%}$/TCP-CPE could be used in similar way and with comparable results. But, there was one distinct difference between, when the latter had not required the removal of oxygen prior to measurement. Thus, the most time-consuming subroutine with bubbling can be eliminated and the whole procedure significantly shortened. In addition, the necessity to purge the solution would require the permanent source of inert gas, which could complicate eventual field (outdoor) monitoring, which, in the case of the Bi/TCP-CPE, becomes irrelevant.

4. Conclusion

In this article, electroanalysis of some neoniciotinoids has been reviewed, for the first time, in exclusive combination with carbon paste-based electrodes. As shown, the electrode of choice can be the TCP-CPE; a special type of carbon paste electrode with tricresyl phosphate as the pasting liquid. Alternatively, one can employ the same electrode as the substrate (support) for either being plated with a bismuth film (with the aid of controlled electrolysis); i.e., the BiF/TCP-CPE configuration, or its related variant prepared again from the bare TCP-CPE when admixing a fine bismuth powder into the bulk; the resultant configuration being $Bi_{5\%}$/TCP-CPE.

The proper determination with both TCP-CPE and Bi(F)/TCP-CPE surprisingly does not involve any accumulation step since the compounds of interest - herein: *Imidacloprid, Nitenpyram*, *Clothianidin* and *Thiamethoxam* - have not exhibited any notable affinity to be spontaneously pre-concentrated at the CP-surface. Despite this rather unusual feature, the determination of the target compounds can be accomplished with good sensitivity, allowing one to detect the analyte(s) of interest down to the low ppm ($\mu g\ mL^{-1}$) level.
One of the main goals of this article was to demonstrate that often underestimated electroanalysis can also be a powerful tool for the determination of some organic pollutants, with its overall performance fully comparable to separation and optical techniques, but applicable at much lower investment and operational costs. Moreover, electroanalytical procedures, including those reviewed herein, have great potential for being used in the field monitoring and in *on-line* analysis of insecticides, including the neonicotinoids.

5. Acknowledgement

Authors acknowledge financial support of the Ministry of Education and Science (Project No. 172012 and 172059), the Secretariat for Science and Technological Development of AP Vojvodina, Republic of Serbia (Grant No. 114-451-02011/2007-02) and CEEPUS II network (CIII-CZ-0212-05-1112). The Czech authors would like to thank the financial support from the Czech Ministry of Education, Youth, and Sports (projects LC 06035 and MSM0021627502).

6. References

Abramović B., et al. (2007). Visible-light-induced photocatalytic degradation of herbicide mecoprop in aqueous suspension of TiO_2. *Acta Chimica Slovenica,* Vol. 54, pp. 558-564

Adams, R.N. (1958). Carbon paste electrodes. *Analytical Chemisty*, Vol. 30, pp. 1576-1576.

Adams, R.N. (1963). Carbon paste electrodes: A Review. *Reviews on Polarography (Kyoto, JPN)*, Vol. 11, pp. 71-78.

Adams, R.N. (1969). *Electrochemistry at solid electrodes*, Dekker, New York

Alaux C., et al. (2010). Interactions between *Nosema* microspores and a neonicotinoid weaken honeybees (*Apis mellifera*). *Environmental Microbiology*, Vol. 12, pp. 774–782

Alizadeh, T. (2009). High selective Parathion voltammetric sensor development by using an acrylic based molecularly imprinted polymer-modified carbon paste electrode. *Electroanalysis,* Vol. 12, pp. 1490-1498

Anderson, D., Gibbs, A. J., (1988). Inapparent Virus Infections and their Interactions in Pupae of the Honey Bee (*Apis mellifera* Linnaeus) in Australia. *Journal og General Virology,* Vol. 69, pp. 1617-1625

Barek, J. et al. (2001). Polarography and voltammetry at mercury electrodes. *CRAC – Critical Reviews in Analytical Chemistry,* Vol. 31, pp. 291-310.

Blanc R., et al. (2000). On the estimate of blanks in differential pulse voltammetric techniques: Application to detection limits evaluation as recommended by IUPAC *Analytica Chimica. Acta* , Vol. 403, pp, 117-123.

Černigoj, U. et al. (2007). Degradation of neonicotinoid insecticides by different advanced oxidation processes and studying the effect of ozone on TiO_2 photocatalysis. *Applied Catalysis B* . Vol. 75, pp. 229–238.

Chadim, P. (1999). Possibilities and limitations of carbon paste electrodes plated with a gold film for voltammetric determination of arsenic. MSc diss. (in Czech), Univ. Pardubice.

Chen, Y.-T. et al. (2007). New capillary electrophoresis / electrochemiluminescence detection system equipped with an electrically heated Ru(bpy)3(2+) / multiwall carbon nanotube paste electrode. *Journal of Chromatography A,* Vol. 1172, pp. 84-91

Cheng, X. et al. (2007). Determination of four kinds of carbamate pesticides by capillary zone electrophoresis with amperometric detection at a polyamide-modified carbon paste electrode. *Talanta*, Vol. 71, (2007), pp. 1083-1087

Ciucu, A.A. et al. (2003). Detection of pesticides using an amperometric biosensor based on ferrophthalocyanine chemically modified carbon paste electrode and immobilized bienzymatic system. *Biosensors and Bioelectronics*, Vol. 18, pp. 303-310

Claux, B., Vittori, O. (2007). Bismuth film electrode as an alternative for mercury electrodes: Determination of azo dyes and application for detection in food stuffs. *Electroanalysis,* Vol. 19 pp. 2243-2246

Cooper, P.A. et al. (2000). Capillary electrochromatography for pesticide analysis: Effects of environmental matrices. *Electrophoresis. Vol.* 21, pp. 1574-1579

Cox-Foster, L. D., et al. (2007). A metagenomic survey of microbes in honey bee colony collapse disorder. *Science*, Vol. 318, pp 283–287

De Erenchun, R. et al. (1997). Determination of imidacloprid and its major metabolite in soils by liquid chromatography with pulsed reductive amperometric detection. *Analytica Chimica Aca,* Vol. 349, pp. 199-206

de Figueiredo-Filho, L.C.S. et al. (2010). Differential pulse voltammetric determination of paraquat using a bismuth-film electrode, *Electroanalysis* , Vol. 22 , pp. 1260-1266

Dell'Arciprete, M.L. et al. (2010). Reactivity of neonicotinoid pesticides with singlet oxygen, *Catalysis Today* Vol. 151, pp. 137-142

Desneux N., A. et al. (2007). The sublethal effects of pesticides on beneficial arthropods. *Annual Review of Entomology,* Vol. 52, pp. 81–106

El Bakouri, H. et al. (2005). Electrochemical analysis of Endosulfan using a C18-modified carbon-paste electrode. *Chemosphere,* Vol. 60, pp. 1565-1571

Fan, S.-H. et al. (2008). Sensitive voltammetric response of methylparathion on single-walled carbon nanotube paste coated electrodes using ionic liquid as binder. *Sensors Actuators B,* Vol. 132, pp. 34-39

Feo, M. L., et al. (2011). Performance of gas chromatography/tandem mass spectrometry in the analysis of pyrethroid insecticides in environmental and food samples. *Rapid Communications in Mass Spectrometry,* Vol. 25, pp. 869–876.

Gaál, F.F. et al. (2007). Determination of various insecticides and pharmaceuticals using differently modified glassy carbon electrodes. *Journal of the Serbian Chemical Society,* Vol. 72, pp. 1465-1475

Gilden, R.C. et al. (2010). Pesticides and health risks, *Journal of Obstetric, Gynecologic, & Neonatal Nursing,* Vol. 39, (2010), pp. 103-110

Girolami, V et al. (2009). Translocation of Neonicotinoid Insecticides from Coated Seeds to Seedling Guttation Drops: A Novel Way of Intoxication for Bees. *Journal of Economic Entomology, Vol. October, pp.* 1808–1815

Gorton, L. (1995). Carbon paste electrodes modified with enzymes, tissues, and cells. A Review. *Electroanalysis*, Vol. 7, pp. 23-45.

Gosser, D.K. Jr. (1993). *Cyclic Voltammetry: Simulation and Analysis of Reaction Mechanisms*, New York: VCH Publishers.

Guiberteau, A. et al. (2001). Study and determination of the pesticide Imidacloprid by square wave adsorptive stripping voltammetry.*Talanta,* Vol. *53*, pp. 943-949.

Gupta, P.K. (2004). Pesticide exposure—Indian scene. *Toxicology,* Vol. 198, (2004), pp. 83–90

Guziejewski, D. (2011b). Application of Catalytic Hydrogen Evolution in the Presence of Neonicotinoid Insecticide Clothianidin, *Food Analytical Methods* , pp. 1-8 , article in press

Guziejewski, D. et al. (2011a). Cathodic stripping voltammetry of clothianidin: Application to environmental studies. *Collection of Czechoslovak Chemical Communications,* Vol 76, pp. 131-142

Guzsvány V, et al. (2006b). ^{1}H NMR Study of the Influence of pH on the Persistence of Some Neonicotinoids in Water. *Acta Chimica Slovenica,* Vol. 53, pp. 52-57.

Guzsvány V. (2007). Bismuth film Electrode for Voltammetric Analysis. *Journal of Flow Injection Analysis*, Vol. 24, pp 126

Guzsvány V. et al. (2006c). Bismuth Film Electrode for the Cathodic Electrochemical Determination of Thiamethoxam. *Electroanalysis,* Vol. 18, pp. 1363-1371

Guzsvány, V. et al. (2008b). Monitoring of Photocatalytic Degradation of Selected Neonicotinoid Insecticides by Cathodic Voltammetry with a Bismuth Film Electrode, *Electroanalysis,* Vol. 20, pp. 291- 300

Guzsvány, V. (2006). Characterisation and determination of selected neonicotinoid insecticides. PhD thesis, University of Novi Sad, Faculty of Sciences

Guzsvány, V. et al. (2005). Voltammetric determination of imidacloprid and thiamethoxam. *Journal of the Serbian Chemical Society,* Vol. 70, pp. 735-743

Guzsvány, V. et al. (2006a). Rapid differential pulse polarographic determination of thiamethoxam in commercial formulations and some real samples. *Microchimica Acta,* Vol. 154, pp. 321-328

Guzsvány, V. et al. (2007a). Determination of Selected Neonicotinoid Insecticides by Liquid Chromatography with Thermal Lens Spectrometric Detection, *Environmental Chemistry Letters,* Vol. 5, pp. 203-208

Guzsvány, V. et al. (2007b). Cathodic Electrochemical Application of Bismuth Film Electrode: Determination of Thiamethoxam Insecticide. U: K. Vytřas, K. Kalcher: *Sensing in Electroanalysis,* Pardubice (Czech Republic): University of Pardubice, ISBN 978-80-7194-954-1. pp 75-84

Guzsvány, V. et al. (2008a). Voltammetric monitoring of photocatalytic degradation of imidacloprid, nitenpyram and acetamiprid neonicotinoid insecticides, U: K. Vytřas, K. Kalcher, eds: *Sensing in Electroanalysis*, Pardubice (Czech Republic): University of pardubice, ISBN 978-80-7395-087-3. pp. 105- 116

Guzsvány, V. et al. (2009a). Rapid spectrophotometric determination of imidacloprid in selected commercial formulations in the presence of 6-chloronicotinic acid. *Journal of Serbian Chemical Society.* Vol. 74, pp. 1455-1465

Guzsvány, V. et al. (2009b). Photocatalytic Degradation of the Insecticide Acetamiprid on TiO_2 Catalyst, *Journal of Brasilian Chemical Society*, Vol. 20, pp. 152- 159

Guzsvány, V. et al. (2010). Comparison of different iron-based catalysts and TiO_2 for photocatalytic decomposition of imidacloprid, *Reaction Kinetics, Mechanisms and Catalysis.* Vol. 99, pp. 225-233

Guzsvány, V. et al. (2011a). Bismuth Modified Carbon-Based Electrodes for the Determination of Selected Neonicotinoid Insecticides, *Molecules,* Vol. 16, pp. 4451-4466

Guzsvány, V. et al. (2011b). Spectroscopic monitoring of photocatalytic degradation of acetamiprid and their degradation intermedier 6-chloronicotinic acid, in press

Hernandez, L. et al. (1993). Voltammetric determination of Methyl parathion, ortho-, meta- and para-nitrophenol with a carbon paste electrode modified with C-18. *Fresenius Journal of Analytical Chemistry,* Vol. 345, pp. 712-715

Hernandez, P. et al. (1993). Use of an electrode of carbon paste modified with C-18 in the determination of Bendiocarb. *Química Analítica (Barcelona)*, Vol. 12, pp. 18-23

Hernandez, P., et al. (1989). Determination of Tetramethrin (Neopynamin) by differential pulse voltammetry with a carbon paste electrode modified with sepiolite. *Fresenius Zeitschrift für analitische Chemie,* Vol. 334, pp. 550-553

Hernández-Borges, J., et al. (2004). Pesticide analysis by capillary electrophoresis. *Journal of Separation Science*, Vol. 27, pp. 947-963

Hočevar, S. et al. (2005). Novel electrode for electrochemical stripping analysis based on carbon paste modified with bismuth powder. *Electrochimica Acta,* Vol. 51, pp. 706-710

Hutton, E.A. et al. (2004). Cathodic electrochemical detection of nitrophenols at a bismuth film electrode for use in flow analysis. *Electroanalysis,* Vol. 16, pp. 1616-1621

Jeschke, P. & Nauen, R. (2008). Neonicotinoids - from zero to hero in insecticide chemistry. *Pest Management Science*, Vol. 64, (2008), pp. 1084-1098

Jeschke, P. et al. (2011). Overwiev of the Status and Global Strategy for Neonicotinoids. *Journal of Agricultural and Food Chemistry,* Vol. 59, pp. 2897-2908

Johnson, R. M., et al., (2009). Changes in transcript abundance relating to colony collapse disorder in honey bees (*Apis mellifera*). *The Proceedings of the National Academy of Sciences Online (*U.S.*)*, Vol. 106, pp. 14790-14795

Kalcher, K. (1985a). Voltammetric determination of trace amounts of gold with a chemically modified carbon paste electrode. *Analytica Chimica Acta* Vol. 177, pp. 175-182

Kalcher, K. (1985b). Voltammetric determination of iodide with ion-exchanger modified carbon paste electrode. *Fresenius Zeitschrift fuer Analytische Chemie,* Vol. 321, pp. 666-670

Kalcher, K. (1990). Chemically modified carbon paste electrodes in voltammetric analysis. *Electroanalysis,* Vol. 2, pp. 419-433

Kalcher, K. et al. (2006). Heterogeneous electrochemical carbon sensors. In: *The Encyclopedia of Sensors, Vol. 4* (C.A. Grimes, E.C. Dickey, M.V. Pishko, Eds.) pp. 283-429. ASP Publishers, Stevenson Ranch (USA).

Kalcher, K. et al. (2009). Electrochemical sensors and biosensors based on heterogeneous carbon materials. *Monatshefte für Chemie*, Vol. 140, pp. 861-889

Kalcher, K. et al.(1995). Sensors based on carbon paste in electrochemical analysis: A Review with particular emphasis on the period 1990-1993. *Electroanalysis* , Vol. 7, pp. 5-22

Kalcher, K., et al. (1987). Extractive preconcentration of gold with carbon paste electrodes modified with organophosphorus compounds. *Zeitschrift fuer Analytische Chemie,* Vol. 327, pp. 513-517

Kamel, A. (2010). Refined Methodology for the Determination of Neonicotinoid Pesticides and Their Metabolites in Honey Bees and Bee Products by Liquid

Chromatography−Tandem Mass Spectrometry (LC-MS/MS). *Journal of Agricultural and Food Chemistry* Vol. 58, pp. 5926-5931

Kamrin, M.A. (1997). *Pesticide Profiles: toxicity, environmental impact, and fate.* CRC Press, ISBN: 978-1-56670-190-197

Kim, H. J. et al. (2003). Enzyme-linked immunosorbent assays for the neonicotinoid insecticides.*Korean Journal of Agricultural Chemistry and Biotechnology,* Vol. 46, pp. 133-136.

Kim, H. J. et al. (2004). Monoclonal antibody-based enzyme-linked immunosorbent assay for the insecticide imidacloprid. *Analytica Chimica. Acta*, Vol. 509, pp. 111-118.

Kim, H.-J., et al. (2003). Development of an enzyme-linked immunosorbent assay for the insecticide thiamethoxam. *Journal of Agricultural and Food Chemistry.* Vol. 51 (7), pp. 1823-1830

Kim, H.-J., et al. (2006). Automated flow fluorescent immunoassay for part per trillion detection of the neonicotinoid insecticide thiamethoxam. *Analytica Chimica Acta. Vol.* 571 , pp. 66-73

Kissinger, P.T. and W.R. Heineman (Eds.) (1996). *Laboratory Techniques in Electroanalytical Chemistry*, Revised and expanded edition. New York: Marcel Dekker.

Komorsky-Lovric S. et al. (2007). *Square-Wave Voltammetry. Theory and Application.* Springer, Berlin.

Konvalina, J. and K. Vytřas (1999). Determination of thallium(III) at a carbon paste electrode using potentiometric stripping analysis. In: *Monitoring of Environmental Pollutants*, (K. Vytřas, J. Kellner, J. Fischer; Ed.) pp. 99-104. University of Pardubice, Pardubice.

Kumaravel, A. and Chandrasekaran, M. (2011). Electrochemical determination of imidacloprid using nanosilver Nafion®/nanoTiO2 Nafion® composite modified glassy carbon electrode. *Sensors and Actuators, B: Chemical*, article in press

Lambropoulou D. A. and Albanis T.A. (2007). Liquid-phase micro-extraction techniques in pesticide residue analysis. Journal of Biochemical and Biophysical Methods, Vol. 70, pp. 195-228

Lee, J. K. et al. (2001). Development of an ELISA for the detection of the residues of the insecticide imidacloprid in agricultural and environmental samples. *Journal of Agricultural and Food Chemistry*. Vol. 49, pp. 2159-2167.

Lei, Y. et al. (2004). Whole cell-enzyme hybrid amperometric biosensor for determination of organo-phosphorous nerve agents with p-nitrophenyl substituent. *Biotechnology and Bioengineering,* Vol. 85, pp. 706-713

Lei, Y. et al. (2005). Highly sensitive and selective amperometric microbial biosensor for direct determination of p-nitrophenyl-substituted organophosphate nerve agents. *Environmental Science & Technology,* Vol. 39, pp. 8853-8857

Lei, Y. et al. (2007). Biosensor for direct determination of Fenitrothion and EPN using recombinant Pseudomonas putida JS444 with surface-expressed organophosphorous hydrolase. 2. Modified carbon paste electrode. *Applied Biochemistry Biotechnology,* Vol. 136, pp. 243-250

Li, K. et al. (2000). Development of an enzyme-linked immunosorbent assay for the insecticide imidacloprid. *Journal of Food and Agricultural Chemistry.* Vol. 48, pp. 3378-3382

Li. D. et al. (2010). A study on the electroanalytical performance of a bismuth film-coated and Nafion-coated glassy carbon electrode in alkaline solutions. *Microchimica Acta,* Vol. 169 pp. 221-225

Lin, Z. et al. (2009).Applications of capillary electrophoresis and capillary electrochromatography in the detection of pesticide residues. *Electrophoresis.* Vol. 21, pp. 1574-1579

Liu, G.-D. & Lin, Y.-H. (2005). Electrochemical stripping analysis of organo-phosphate pesticides and nerve agents. *Electrochemistry Communications,* Vol. 7, pp. 339-343

Liu, S. et al. (2010). Simultaneous determination of seven neonicotinoid pesticide residues in food by ultraperformance liquid chromatography tandem mass spectrometry. *Journal of Food and Agricultral Chemistry*, Vol. 58, pp. 3271-3278

Ly, S.-Y. et al. (2008). Administering pesticide assays in in-vivo implanted biosensors. *Australian Journal of Chemistry,* Vol. 61, pp. 826-832

Majidi, M.R. et al. (2011), Determination of Imidacloprid in tomato grown in greenhouse based on copper(II) phthalocyanine modified carbon ceramic electrode by differential pulse voltammetry*, Journal of the Chinese Chemical Society. Vol.* 58, pp. 207-214

Malato, S. et al. (2003). Applied studies in solar photocatalytic detoxification: an overview. *Solar Energy*, Vol. 75, pp. 329-336.

Malato, S. et al. (2001). Degradation of imidacloprid in water by photo-Fenton and TiO_2 photocatalysis at a solar pilot plant: a comparative study, *Environ. Sci. Technol.* Vol. 35, pp. 4359–4366

Malato, S. et al., (2002). Photocatalytic treatment of water-soluble pesticides by photo-Fenton and TiO_2 using solar energy. *Catalysis Today*. Vol. 76, pp. 209–220

Mandić, I. A. et al. (2005). Determination of the insecticide imidacloprid in potato (Solanum tuberosum L.) and onion (Allium cepa) by high-performance liquid chromatography with diode-array detection. *Journal of Analytical Chemistry,* Vol. 60, pp. 1134–1138

Moreno, M. et al. (2009). Cathodic electrochemical determination of herbicides in acid media using a bismuth film electrode, *Electroanalysis*, Vol. 21, pp. 415-421

Mulchandani, P. et al. (2001a). Flow injection amperometric enzyme biosensor for direct determination of organophosphate nerve agents. *Environmental Science and Technology,* Vol. 35, pp. 2562-2565

Mulchandani, P. et al. (2001b). Amperometric microbial biosensor for direct determination of organophosphate pesticides using recombinant microorganism with surface expressed organophosphorus hydrolase. *Biosensors and Bioelectronics,* Vol. 16, pp. 433-437

Nauen, R. et al. (2008). In focus: neonicotinoid insecticides. Pest Management Science, Vol. 64, p. 1081

Navalón, A. et al. (1999). Differential-pulse polarographic determination of the insecticide imidacloprid in commercial formulations. *Mikrochimica Acta,* Vol. 130, pp. 261-265

Nigović, B. et al. (2009). Voltammetric measurements of aminosalicylate drugs using bismuth film electrode, *Electrochimica Acta* , Vol. 54 , pp. 5678-5683

Obana, H. et al. (2002). Determination of acetamiprid, imidacloprid and nitenpiramresidues in vegetables and fruits by high-performance liquid chromatography with diode-array detection. *Journal of Agricultural and Food Chemistry,* Vol. 50, pp. 4464–4467

Obana, H. et al. (2003). Determination of neonicotinoid pesticide residues in vegetables and fruits with solid phase extraction and liquid chromatography-mass spectrometry, *Journal of Agricultural and Food Chemistry,* Vol. 51, pp. 2501-2505.

Papp, Zs. (2011). Voltammetric characterization and determination of selected neonicotinoid insecticides using different carbon-based electrodes, PhD thesis, University of Novi Sad, Faculty of Sciences.

Papp, Zs. et al. (2009a). New applications of tricresyl phosphate-based carbon paste electrodes in voltammetric analysis. In: *Sensing in Electroanalysis*, *Vol. 4.*, K. Vytřas, K. Kalcher, and I. Švancara (Ed.), 47-58. Univ. Press Center, ISBN 978-80-7395-212-9, Pardubice, Czech Republic

Papp, Zs. et al. (2009b). Voltammetric determination of Imidacloprid insecticide in selected samples using carbon paste electrodes. *Microchimica Acta,* Vol. 166, pp. 169-175

Papp, Zs. et al. (2011). Carbon paste electrodes for the analysis of some agricultural pollutants and trace metals, *Journal of Agricultural Science and Technology,* Vol. 5, pp. 85-92, ISSN 1939-1250

Papp, Zs.J. et al. (2010). Voltammetric determination of the neonicotinoid insecticide Thiamethoxam using a tricresyl phosphate-based carbon paste electrode. *Journal of Serbian Chemical Society,* Vol. 75, pp. 681-687

Parham, H. & Rahbar, N. (2010). Square-wave voltammetric determination of Methyl-parathion at ZrO_2-nanoparticles modified carbon paste electrode. *Journal of Hazardous Materials,* Vol. 177, pp. 1077-1084

Pesticide, 2011, http://en.wikipedia.org/wiki/Pesticide

Picó, Y. et al. (2003) Capillary electrophoresis for the determination of pesticide residues *TrAC - Trends in Analytical Chemistry*, Vol. 22, pp. 133-151

Quintas, G. et al. (2004). Fourier transform infrared determination of imidacloprid in pesticide formulations. *Journal of Brazilian Chemical Society*, Vol. 15, pp. 307-312

Rancan, M. et al. (2006a). Determination of Imidacloprid and metabolites by liquid chromatography with an electrochemical detector and post column photochemical reactor. *Analytica Chimica Acta*, Vol. 555, pp. 20-24

Rancan, M. et al. (2006b). Determination of Thiamethoxam residues in honeybees by high performance liquid chromatography with an electrochemical detector and post-column photochemical reactor. Journal of Chromatography A, Vol. 1123, pp. 60-65

Ridding the World of POPs: A guide to the Stockholm Convention on Persistent Organic Pollutants (2005) http://www.pops.int/documents/guidance/beg_guide.pdf

Rocío, C. et al. (2011). Single solid phase extraction method for the simultaneous analysis of polar and non-polar pesticides in urine samples by gas chromatography and ultrahigh pressure liquid chromatography coupled to tandem mass spectrometry. *Talanta,* Vol. 85, pp. 183-196

Schultz, R. et al. (2003) Fate and effects of azinphos-methyl in a flow-through Wetland in South Africa Peall, *Environmental Science & Technology,* Vol. 37, (2003), pp. 2139-2144

Shaidarova, L.G. et al. (1998). Stripping voltammetry of biologically active organic pollutants as host-guest complexes at electrodes modified with crown ether. *Journal of Analytical Chemisty,* Vol. 53, pp. 52-58

Shankaran, D. et al. (2007) Recent advancements in surface plasmon resonance immunosensors for detection of small molecules of biomedical, food and environmental interest. *Sensors and Actuators B: Chemical*, Vol. 121, pp. 158-177

Sirisha, K. et al. (2007). Differential pulse adsorptive stripping voltammetric determination of Chlorpyrifos at a sepiolite modified carbon paste electrode. *Analytical Letters,* Vol. 40, pp. 1939-1950

Skladal, P. (1991). Determination of organophosphate and carbamate pesticides using a cobalt phthalocyanine-modified carbon paste electrode and a cholinesterase enzyme membrane. *Analytica Chimica Acta,* Vol. 252, pp. 11-15

Srey, M. (1992). Voltammetric and potenciometric method for the determination of gold. MSc diss. (in Czech), Univ. Chem. Technol., Pardubice.

Suchail, S. et al. (2000). Characteristics of imidacloprid toxicity in two Apis mellifera subspecies. *Environmental Toxicology & Chemistry,* Vol. 19, pp. 1901–1905.

Švancara I. et al. (2001b) A Study on the applicability of carbon paste electrodes for the determination of 6-benzylaminopurine. *Scientific Papers of the University of Pardubice, Series A* Vol. 7, pp. 17-32

Švancara, I. and Schachl, K. (1999). Testing of unmodified carbon paste electrodes: A Review. *Chemicke Listy*, Vol. 93, pp. 490-499

Švancara, I. and Vytřas, K. (1993) Voltammetry with carbon paste electrodes containing membrane plasticizers used for PVC-based ion-selective electrodes. *Analytica Chimica Acta, Vol.* 273, pp. 195-204

Švancara, I. et al (2011). *Electroanalysis with Carbon Paste Electrodes*. Taylor & Francis / CRC Press, Boca Raton (FL, USA), in press

Švancara, I. et al. (1994). Voltammetric investigations on carbon paste electrodes as supports for mercury films. *Electroanalysis.* Vol. 6, pp. 663-671

Švancara, I. et al. (1996a). A microscopic study on carbon paste electrodes. *Electroanalysis*, Vol. 8, pp. 61-65

Švancara, I. et al. (2001a) Carbon paste electrodes in modern electroanalysis (A Review). *CRAC – Critical Reviews of Analytical Chemistry,* Vol 31, pp. 311-345

Švancara, I. et al. (2002). Simple and rapid determination of iodide in table salt by stripping potentiometry at a carbon paste electrode. *Analytical and Bioanalytical Chemistry, Vol.* 372, pp. 795-800

Švancara, I. et al. (2009a). Carbon paste electrodes in facts, numbers, and notes: A Review on the occasion of the 50-years jubilee of carbon paste in electrochemistry and electroanalysis. *Electroanalysis,* Vol. 21, pp. 7-28

Švancara, I. et al. (2010). A decade of bismuth-modified electrodes in electroanalysis (A review). *Electroanalysis,* Vol. 22, pp. 1405-1420

Švancara, I. et al. 2009b. Carbon paste electrodes in the new millennium (A review). *CEJC–Central European Journal of Chemistry*, Vol. 7, pp. 598-656

Švancara, I. et al.(2006). Recent advances in anodic stripping voltammetry with bismuth-modified carbon paste electrodes. *Electroanalysis,* Vol. 18, pp. 177-185

Švancara, I., et al. (1996b). Voltammetric determination of silver at ultratrace levels using a carbon paste electrode with improved surface characteristics. *Electroanalysis,* Vol. 8, pp. 336-342

Švancara, I., et al. (1998a). Stripping voltammetric determination of iodide with synergistic accumulation at a carbon paste electrode. *Electroanalysis,* Vol. 10, pp. 435-441

Švancara, I., et al. (1998b). The testing of carbon paste electrodes: An example on the characterisation of a carbon paste electrode prepared from newly used graphite powder. *Scientific Papers of the University of Pardubice, Series A,* Vol. 4, pp. 49-63

Tan, X.-H. et al. (2010). Sensitive voltammetric determination of Methylparathion using a carbon paste electrode modified with mesoporous zirconia. *Electroanalysis,* Vol. 22, pp. 151-154

Tapparo A. et al. (2011). Rapid analysis of neonicotinoid insecticides in guttation drops of corn seedlings obtained from coated seeds, *Journal of Environmental Monitoring,* DOI: 10.1039/c1em10085h

Tuzimski, T. (2011). Multidimensional Chromatography in Pesticides Analysis, In: *Pesticides - Strategies for Pesticides Analysis*, M. Stoytcheva, (Ed.), 155-196, InTech, ISBN 978-953-307-460-463

Ulakhovich, N.A et al. (1993). Carbon-paste electrodes as chemical sensors in voltammetry. *Zhurnal of Analiticheskoi Khimii,* Vol. 48, pp. 980-998

Ulakhovich, N.A. et al. (1998). Voltammetric determination of Phosalone and Carbophos pesticides using a modified carbon-paste electrode. *Journal of Analytical Chemisty,* Vol. 53, pp. 147-150

Van Dyk J. S., Pletschke B. (2011). Review on the use of enzymes for the detection of organochlorine, organophosphate and carbamate pesticides in the environment. *Chemosphere*, Vol. 82, pp. 291-307

Vilchez, J. L. et al. (1998) . Photochemical-fluorimetric method for the determination of the insecticide imida-cloprid in water samples. *Analytica Chimica Acta*, Vol. 371, pp. 247-253.

Vilchez, J. L. et al. (2001). Flow injection analysis of the insecticide imidacloprid in water samples with photochemically induced fluorescence detection *Analytica Chimica Acta*, Vol. 439, pp. 299–305.

Vytřas, K. (1985) Potentiometric titrations based on ion-pair formation. 1985. *Ion-Selective Electrode Reviews*, Vol. 7 pp. 77-164.

Vytřas, K. et al. (1993). Voltammetric and potentiometric determination of gold in gold-plated electrotechnical components. *Collections of Czechoslovak Chemical Communications,* Vol. 58, pp. 2039-2046.

Vytřas, K., I. Švancara. (2007). Carbon paste-based ion-selective electrodes. In: *Sensing in Electroanalysis, Vol. 2 (*K. Vytřas, K. Kalcher, Eds.), pp. 7-22. Univ. Press Pardubice, Pardubice.

Wang, J. (1985). Stripping analysis: Principles, instrumentation, and applications. Orig. Ed.,. Deerfield Beach: VCH Publishers.

Wang, J. (2002). Real-time electrochemical monitoring: Toward green analytical chemistry. *Assesments in Chemical Research,* Vol. 35, pp. 811-816.

Wang, J. (2005). Stripping analysis at bismuth electrodes: A review. *Electroanalysis, Vol.* 17, pp. 1341-1346.

Watanabe E. et al. (2004b). Rapid and simple screening analysis for residual imidacloprid in agricultural products with commercially available ELISA. *Analytica Chimica Acta*, Vol. 521, pp. 45-51

Watanabe, E. et al. (2004a). Evaluation and validation of a commercially available enzyme-linked immunosorbent assay for the neonicotinoid insecticide imidacloprid in agricultural samples. *Journal of Agricultural and Food Chemistry,* Vol. 52, pp. 2756-62.

Watanabe, E. et al. (2006). Immunoassay for acetamiprid detection: application to residue analysis and comparison with liquid chromatography. *Analytical and Bioanalytical Chemistry*, Vol. 386, 1441–1448

Watanabe, E. et al. (2007). Simultaneous determination of neonicotinoid insecticides in agricultural samples by solid-phase extraction cleanup and liquid chromatography equipped with diode-array detection. *Journal of Food and Agricultral Chemistry*, Vol. 55, pp. 3798-3804

Watanabe, S. et al. (2001). Development of competitive enzyme-linked immunosorbent assays (ELISAs) based on monoclonal antibodies for chloronicotinoid insecticides imidacloprid and acetamiprid. *Analytica Chimica Acta*, Vol. 427, pp. 211-219

Yang, C. E. et al., (2008). Abnormal foraging behavior induced by sublethal dosage of imidacloprid in the honey bee (Hymenoptera: Apidae). *Journal of Economic Entomology,* Vol. 101, pp. 1743–1748

Zima, J. et al. (2009a). Recent advances in electroanalysis of organic and biological compounds at carbon paste electrodes. *CrAC – Crit. Rev. Anal. Chem.* Vol. 39, pp. 204-227.

Zima, J. et al. (2009b). Carbon paste electrodes for the determination of detrimental substances in drinking water. In *Progress on drinking water research, Chapter 1*; (M.H. Lefebvre and M.M. Roux, Eds.) pp. 1-54. Nova Science Publishers, Hauppauge (USA).

25

Insecticide Activity of Lectins and Secondary Metabolites

Patrícia M.G. Paiva[1], Thiago H. Napoleão[1], Roberto A. Sá[2] and Luana C.B.B. Coelho[1]
[1]*Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Pernambuco, Av. Prof. Moraes Rego, S/N, 50670-420, Recife-PE,*
[2]*Centro Acadêmico do Agreste, Universidade Federal de Pernambuco, 55002-970, Caruaru-PE,*
Brazil

1. Introduction

Proteins are polymers of amino acids (molecules containing an amino group, a carboxylic group and a hydrophobic or hydrophilic side chain) present in all organisms. Apolar, polar uncharged and electrically charged amino acids are covalently linked through peptide bonds (amide bonds) and the sequence they form in the polypeptide chain (primary structure) determines the tertiary or quaternary structures ultimately presenting some biological activity. Proteins can be formed by one or multiple polypeptides (subunits) with or without a non-amino acid molecule (carbohydrate, ion, lipid, etc) linked to them.

Lectins comprise a heterogeneous group of non-immune proteins that interact with carbohydrates. This interaction is behind a number of biological properties, including antimicrobial, antitumoral, hemagglutinating, mitogenic and insecticide activities.

The specificity of the carbohydrate binding site is determined by the amino acids forming the lectin molecule, as well as shape and the spatial arrangement of neighboring amino acids; additionally, metal ions may contribute for correct positioning of the amino acid residues for binding to the carbohydrate (Sharon and Lis, 2001). Lectins can be divided into those that bind monosaccharides as well as oligosaccharides, and those that recognize only oligosaccharides (Sharon and Lis, 2007). Depending on carbohydrate specificity, they can be classified as: glucose/mannose, *N*-acetylglucosamine, galactose, *N*-acetylgalactosamine, fucose and sialic acid-binding lectins (Wu et al., 2001). The hemagglutinating activity assay (Figure 1A) in presence of free carbohydrates (Figure 1B) has been proved to be a useful tool to characterize lectin specificity.

Plant lectins have been isolated from bark, cladodes, flowers, leaves, rhizomes, roots and seeds. They differ from each other with respect to their molecular structures, carbohydrate-binding specificities, and biological activities. The compact globular structures, molecular aggregation and glycosylation of lectins in general result in high structural stability (Kawsar et al., 2008; Moreno et al., 2008).

In general, lectin isolation procedures include protein extraction steps with aqueous solvent, the production of a lectin-rich fraction, and separation of lectin from protein or non-protein

contaminants by chromatography. Lectin solubility and stability vary with the sequence of amino acids in the polypeptide chain, and such structural features can be exploited to provide concentrated lectin preparations. Lectins can be precipitated from extracts by adding ammonium sulfate at high concentration (salting out method) or organic solvents (Santana et al., 2008; Napoleão et al., 2011). Heat-stable lectins can be partially purified by submitting the extract to high temperature for removal of other proteins (Santana et al., 2008). Lectins are purified by ion exchange, molecular exclusion and/or affinity chromatography that rely on characteristics like charge, size and biological affinity of lectin for solid phases, respectively.

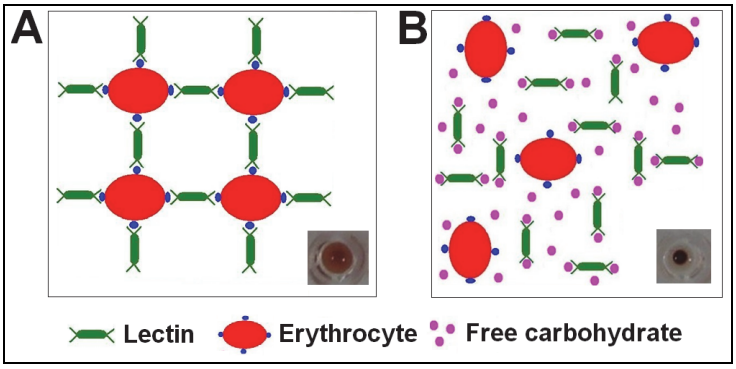


Fig. 1. Schematic representation of erythrocyte network promoted by lectin binding to surface carbohydrates (A) and inhibition of hemagglutinating activity by free carbohydrate (B). Aspects of assays in microtiter plates (insets).

Isolated lectin can be detected by polyacrylamide gel electrophoresis (PAGE) using dyes such as Coomassie Brilliant Blue or Amido black (Reisfeld et al., 1962; Laemmli, 1970). Specific staining techniques with Schiff's reagent (Pharmacia Fine Chemicals, 1980) or Concanavalin A-Peroxidase (Hinata and Nishio, 1981) can easily reveal the presence of glycan; carbohydrate moiety characterization can be performed after lectin tryptic digestion in gel followed by enzymatic deglycosylation and mass spectrometric analysis (Nasi et al., 2009).

1.1 Lectins from *Bauhinia monandra* leaf and secondary roots

Bauhinia monandra (Angiosperms, Eudicots, Rosids, Eurosids I/Fabidae, Order Fabales, Family Fabaceae) has the popular names "pata-de-vaca" in Portuguese, "orquidea del pobre" in Spanish, and pulse or Napoleon's plume in English (Judd et al., 2007; Souza et al., 2011b). *B. monandra* leaf infusions are used as medicine in the treatment of diabetes mellitus.

Two lectins were purified at milligram level from leaf and secondary roots of *B. monandra*, and were called BmoLL and BmoRoL, respectively. The isolation procedures included protein extraction with 0.15 M NaCl, ammonium sulphate (60%) fractionation and affinity chromatography on guar gel column (Coelho and Silva, 2000; Souza et al., 2011b).

BmoLL agglutinated rabbit and human (AB and B types) erythrocytes and this hemagglutinating activity was inhibited by D(+)galactose and D(+)rafinose. It was detected over a broad pH range, being heat stable up to 50 ^{o}C (Coelho and Silva, 2000).

Polyacrylamide gel electrophoresis for denatured proteins (SDS-PAGE) revealed that BmoLL is formed by two polypeptides (a 26-kDa subunit and a 33-kDa glycosylated subunit). This lectin did not induce genotoxic effects in a series of cell-free and bacterial assays (Sisenando et al., 2009).
BmoRoL showed hemagglutinating activity on human and rabbit erythrocytes at a pH range of 6.5 to 7.5, and was active up to 60 ºC, losing its activity above this temperature. SDS-PAGE revealed that the lectin was a 26-kDa glycoprotein. BmoRoL showed antifungal activity against *Fusarium solani* and *F. oxysporum* (Souza et al., 2011b).

1.2 Lectin from *Opuntia ficus indica* cladodes

Opuntia ficus indica Mill. (Angiosperms, Eudicots, Order Caryophyllales, Family Cactaceae) has the popular names "palma forrageira" or "figo-da-Índia" in Portuguese, "nopal" or "tuna" in Spanish, and Indian fig opuntia or barbary fig in English (Judd et al., 2007). Cladodes are used in folk medicine and studies demonstrated their diuretic, antiulcer and wound-healing activities (Galati et al., 2001; Galati et al., 2002; Trombetta et al., 2006). *O. ficus indica* is grown in northeastern Brazil as an important feed source for animals, and cladodes have been reported to be a component in sheep feed (Tegegne et al., 2007).
The procedure for isolation of *O. ficus indica* lectin (OfiL) included protein extraction with 0.15 M NaCl and chromatography of extract on a chitin column. OfiL agglutinated rabbit, chicken or human erythrocytes. The hemagglutinating activity was inhibited by monosaccharides and glycoproteins, stimulated by Ca^{2+} or Mg^{2+}, remaining stable across wide pH and temperature ranges. SDS-PAGE revealed that lectin is a single 8.4-kDa polypeptide. OfiL showed antifungal activity against *Colletotrichum gloeosporioides*, *Candida albicans*, *Fusarium decemcellulare*, *Fusarium lateritium*, *Fusarium moniliforme*, *Fusarium oxysporum* and *Fusarium solani*. This lectin was mainly active on *C. albicans* (Santana et al., 2009).

1.3 Lectins from *Moringa oleifera* seeds

Moringa oleifera (Angiosperms, Eudicots, Rosids, Eurosids II/Malvidae, Order Brassicales, Family Moringaceae) has the popular names ''moringa" in Portuguese, ''árbol del ben" in Spanish, and horseradish tree in English (Judd et al., 2007). The seeds are widely used in developing countries as a natural coagulant to treat water for human consumption. It has been demonstrated that a 3-kDa organic polyelectrolyte and proteins with molecular mass of 6.5 to 13 kDa and isoelectric points between 9.6 and 11.0 have coagulant properties (Gassenschmidt et al., 1995; Ndabigengesere et al., 1995; Okuda et al., 2001; Ghebremichael et al., 2005).
Santos et al. (2005) revealed the presence of water-soluble *M. oleifera* lectin (WSMoL) in *M. oleifera* seed extracts by detection of hemmaglutinating activity. The procedure for WSMoL isolation was defined by Coelho et al. (2009) and included the steps of protein extraction with water, precipitation of lectin with ammonium sulfate (60% saturation) and chromatography of precipitated fraction on a chitin column. WSMoL agglutinated human and rabbit erythrocytes in a broad pH range of 4.5 to 9.5 and when kept at 100 ºC for 5 h plus incubation overnight at 37 ºC. The carbohydrate binding site of lectin recognized D(+)-fructose and *N*-acetylglucosamine, since these monosaccharides inhibited the hemagglutinating activity (Rolim et al., 2011). MALDI-TOF/TOF analysis revealed that WSMoL showed similarity with *M. oleifera* protein (Coelho et al., 2009). Genotoxicity assessment of WSMoL using the cell-free plasmid DNA as well as the Ames and Kado

assays showed that this lectin was nonmutagenic (Rolim et al., 2011). WSMoL showed coagulant and antibacterial activities against *Escherichia coli*, *Staphylococcus aureus* and natural lake water bacteria (Ferreira et al., 2011).
The procedure for isolation of coagulant *M. oleifera* lectin (cMoL) was defined by Santos et al. (2009) and included the steps of protein extraction with 0.15 M NaCl, precipitation of lectin with 60% ammonium sulfate and chromatography of precipitated fraction on guar gel column. cMoL agglutinated human and rabbit erythrocytes in a broad pH range of 4.0 to 9.0 and when kept at 100 ºC for 7 h. The hemagglutinating activity of cMoL was inhibited by several carbohydrates, but not by D(+)-fructose. SDS-PAGE revealed that cMoL had a main 26.5-kDa polypeptide band. cMoL showed coagulant property and the ability to bind humic acid, which is interesting when the aim is to remove humic acids from water (Santos et al., 2009; Santos et al., 2011a; Santos et al., 2011b).

1.4 Lectins from *Myracrodruon urundeuva* bark, heartwood and leaf

Myracrodruon urundeuva (Angiosperms, Eudicots, Rosids, Eurosids II/Malvidae, Order Sapindales, Family Anacardiaceae) has the popular names "urundel" in Spanish, pepper tree in English and "aroeira do sertão" in portuguese (Leite, 2002; Judd et al., 2007). The plant has great importance in traditional medicine. Aqueous extracts of the bark showed anti-ulcer and anticholinergic, inflammatory, antidiarrhoeal and analgesic activities (Rao et al., 1987; Almeida-Cortez et al., 2007). *M. urundeuva* heartwood is excellent for poles, fences, pillars, beams, frames, bridges, mills, rafters, parquet, flooring, roofing and turned parts (Mainieri and Chimelo, 1989). Paes et al. (2002) showed that *M. urundeuva* heartwood was resistant to fungi (*Postia placenta* and *Neolentinus lepideus*) and termite (*Nasutitermes corniger*).
M. urundeuva bark, heartwood and leaf are sources of lectins called MuBL, MuHL and MuLL, respectively. The procedures for isolation of these lectins included protein extraction by 0.15 M NaCl, precipitation of lectins with ammonium sulphate (at different saturations for each lectin) and chromatography on a chitin column. The three lectins agglutinated human and rabbit erythrocytes in a broad pH range, and the hemagglutinating activities were inhibited by *N*-acetylglucosamine. SDS-PAGE revealed that MuBL, MuHL and MuLL are polypeptides of 14, 14.4 and 14.2 kDa, respectively (Sá et al., 2009c; Napoleão et al., 2011). MuHL showed antimicrobial activity inhibiting the growth of numerous bacteria (*Bacillus subtilis*, *Corynebacterium callunae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus faecalis*) and fungi *(Fusarium oxysporum, F. decemcellulare*, *F. fusarioides*, *F. solani* and *F. verticilioides* (Sá et al., 2009b).

2. Insecticidal activity of lectins

Lectins have deleterious effects against larvae, developing stages and mature forms of insects from orders Coleoptera, Diptera, Hemiptera, Homoptera, Hymenoptera, Isoptera, Lepidoptera and Neuroptera (Murdock et al., 1990; Eisemann et al., 1994; Powell et al., 1995; Zhu-Salzman et al., 1998; Bandyopadhyay et al., 2001; Isidro et al., 2001; Hogervorst et al., 2006; Kaur et al., 2006; Coelho et al., 2007; Macedo et al., 2007; Fitches et al., 2008; Sá et al., 2008; Coelho et al., 2009; Sá et al., 2009c; Silva et al., 2009; Napoleão et al., 2011; Oliveira et al., 2011; Souza et al., 2011b). Insecticide activity of lectin is generally evaluated by bioassays that incorporate the lectin into artificial diets offered to insects, with insects dying from nutritional deprivation. It has been shown that lectins are resistant to proteases present in the insect gut, a property responsible for their active presence in the digestive tract,

eventually with insecticide effects (Macedo et al., 2007; Napoleão et al., 2011; Oliveira et al., 2011).

The precise mechanisms of insecticidal action of lectins remain unknown, though it has been suggested that this entomotoxic activity seems to depend upon the carbohydrate recognition property they exhibit. Plant lectins with affinity for *N*-acetylglucosamine and chitin-binding property are able to bind chitin and glycosylated proteins of the peritrophic matrix, interfering in the digestion and absorption of nutrients (Tellam et al., 1999; Peumans and Van Damme, 1995; Zhu-Salzman et al., 1998; Zhu-Salzman and Salzman, 2001; Carlini and Grossi-de-Sá, 2002; Macedo et al., 2004; Macedo et al., 2007). The peritrophic matrix constitutes a membrane found in the midgut that separates the contents of the gut lumen from the digestive epithelial cells. The matrix contains a network composed by chitin (polymer of *N*-acetylglucosamine) and glycoproteins such as peritrophins. The importance of the integrity of the peritrophic matrix lies in the protection it offers to midgut epithelial cells against microorganism infection and mechanical damage by abrasive food particles, as apart from the compartmentalization of digestive processes (Hegedus et al., 2009).

Ultrastructural studies have shown abnormalities caused by *Triticum vulgaris* lectin in midgut of *Ostrinia nubilalis* and *Drosophila* such as hypersecretion of many disorganized layers of peritrophic matrix and morphological changes of microvilli (Harper et al., 1998; Li et al., 2009; Vandenborre et al., 2011). Lectin may also cross the midgut epithelial barrier by transcytosis, entering the insect circulatory system and resulting in a toxic action against endogenous lectins involved in haemolymph self-defense mechanisms (Fitches et al., 2001). Lectin may be internalized by endocytotic vesicles into the epithelial cells, blocking nuclear localization and nuclear sequence-dependent protein import, thus inhibiting cell proliferation (Yu et al., 1999).

2.1 Larvicidal activity of lectins against *Callosobruchus maculatus* and *Zabrotes subfasciatus*

Bruchid beetles (Family Chrysomelidae, Subfamily Bruchinae) are small insects under 1 cm in size mainly known for the damage they cause to leguminous seeds. *Callosobruchus* is a cosmopolitan genus behind lowered seed weight, germination viability and marketability, since eggs are laid attached to beans; larvae and pupae develop inside the seeds, which can be attacked both in the field and in storage (Edvardsson and Tregenza, 2005; Souza et al., 2011a). *C. maculatus* (cowpea weevil) is among the main pests of stored cowpea, *Vigna unguiculata* (Angus et al., 2011). Synthetic chemicals, grain protectants and fumigants are extensively used to control insect pests in stored grains; however, the usage of chemical insecticides leads to insecticide residues in grains, and has promoted the emergence of selected resistant populations (Loganathan et al., 2011).

Another important species of bruchid beetles is *Zabrotes subfasciatus* (Mexican bean weevil), which is native to Central and South America. It is one of the main pests of stored beans (*Phaseolus vulgaris*) in Brazil. The females of *Z. subfasciatus* are able to oviposit on the seeds after dehiscence or even when they are already inside the pods, which they enter through perforations (Credland and Dendy, 1992; Sari et al., 2003).

BmoLL showed deleterious effects against *C. maculatus* and *Z. subfasciatus* larvae (Table 1). An artificial seed containing 0.32% BmoLL promoted 50% mortality of *C. maculatus* larvae, while a 50% mass decrease was detected in larvae reared on a diet with seeds containing 0.4% BmoLL. Considering *Z. subfasciatus*, 50% mortality and 20% mass decrease were detected when larvae fed on artificial seeds containing 0.5% BmoLL (Macedo et al., 2007).

Additional assays revealed the ability of BmoLL to bind to a chitin column, the resistance of lectin to digestion by enzymes from *C. maculatus* and *Z. subfasciatus* larvae, the ability of BmoLL-Sepharose column to bind to proteins from midgut homogenates, and the inhibition of α-amylase activity from midgut by BmoLL. Based on these data, it was suggested that the larvicidal activity may be due to BmoLL binding to chitin from gut structures, cell surface glycosylated receptor or sugar moiety of glycoproteins, resistance of lectin to proteolysis by midgut enzymes, and a damaging effect on the digestive enzyme activity (Macedo et al., 2007).

Lectin source and abbreviation	Insect	Damage
Bauhinia monandra leaf (BmoLL)	*Callosobruchus maculatus*	Mortality of larvae; decreased larval weight; decreased α-amylase activity.
	Zabrotes subfasciatus	Mortality of larvae; decreased larval weight.
	Ephestia kuehniella	Decreased larval weight.
B. monandra secondary roots (BmoRoL)	*Nasutitermes corniger*	Mortality of workers and soldiers after ingestion.
Opuntia ficus indica cladodes (OfiL)	*N. corniger*	Mortality of workers and soldiers after ingestion.
Moringa oleifera seeds WSMoL	*Aedes aegypti*	Mortality of fourth-stage larvae (L_4); increased gut volume; disruption of gut underlying epithelium
	N. corniger	Mortality of workers and soldiers after ingestion.
cMoL	*E. kuehniella*	Decreased larval weight; delayed development; mortality of pupae; decreased adult emergence.
	N. corniger	Mortality of workers after ingestion.
Myracrodruon urundeuva Bark and heartwood (MuBL and MuHL)	*A. aegypti*	Mortality of L_4.
MuBL, MuHL and leaf lectin (MuLL)	*N. corniger*	Mortality of workers and soldiers after ingestion; bacteriostatic and bactericide effect against gut symbionts.

References: Macedo et al. (2007); Sá et al. (2008); Sá et al. (2009c); Coelho et al. (2009); Napoleão et al. (2011); Oliveira et al. (2011); Paiva et al. (2011); Souza et al. (2011b).

Table 1. Insecticidal activity of lectins.

2.2 Larvicidal and pupicidal activities of lectins against *Ephestia* (*Anagasta*) *kuehniella*

Ephestia kuehniella (many times referred to as *Anagasta kuehniella*, currently being *Anagasta*, ranked as a subgenus of *Euphestia*) is a moth belonging to the Pyralidae family, and today is a worldwide pest of stored grains, nuts, and legumes. It is commonly found in flour mills. Popularly known as flour moth, *E. kuehniella* also feeds on wheat flour, corn meal, seeds, dried fruits, pasta, baked goods, cocoa, and other stored foods (Gallo et al., 2002; Macedo et al., 2003; Tounsi et al., 2005). Its life cycle lasts 3-4 months and comprises egg, larvae, pupa and adult stages. The larvae (caterpillars) infest the stored product and are the most damaging stage. They produce silk building webs and cocoons in which they complete their development. Next, pupation occurs in the same site. The adults live approximately 14 days and do not feed (Bennett, 2003).

The effect of BmoLL against *E. kuehniella* was determined in a study using an artificial diet containing lectin concentrations of 0.25%, 0.5% or 1.0%. The moths were fed and the mass and number of neonate larvae (fourth instar) were determined (Macedo et al., 2007). The data showed that BmoLL up to 1% did not decrease the survival of larvae, though it produced a 40 % weight decrease (Table 1). The authors reckoned that for every 1% point increase in BmoLL dose, mass decreased by 0.61 mg. BmoLL was resistant to hydrolysis by *E. kuehniella* midgut extracts for 48 h.

The evaluation of insecticidal action of cMoL against *E. kuehniella* used neonate first instar larvae and artificial diet containing 0.5%, 1.0% or 2.0% cMoL. The effect of lectin was determined based on the parameters: weight and number of fourth instar larvae, weight of pupae, time at which the adults emerged and number of adults that emerged (Oliveira et al., 2011). cMoL reduced larval weight, delayed the larval development time by 15 days, promoted pupal mortality and produced low rates of adult emergence, though it did not interfere in larval survival (Table 1). The same study also reported the resistance of cMoL to proteolysis by *E. kuehniella* midgut enzymes.

2.3 Termiticidal activity of lectins against *Nasutitermes corniger*

The tropicopolitan genus *Nasutitermes* (Termitidae family) includes arboreal wood-feeding termites that build their nests in roofs, linings, and structural spans as well as on the soil or above its level (Edwards and Mill, 1986; Scheffrahn et al., 2002). Soldiers of all *Nasutitermes* are easily identified by the dark-brown color of their heads and the characteristically conical nasus that emits a defensive secretion, as well as the presence of six erect setae projecting from the vertex (Scheffrahn et al., 2002). One of the most dominant and broadly distributed species is *N. corniger*. These termites are able to invade the urban environment, attacking wood in the structures of buildings (Scheffrahn et al., 2005; Paes et al., 2007).

Termiticidal activity of lectins (Table 1) has been evaluated by a no-choice bioassay (Figure 2). Briefly, a filter paper disk impregnated with lectin solution or 0.15 M NaCl (negative control) is placed in petri plates. Workers and soldiers are transferred to each plate and the rate of insect survival is determined daily, upon the death of all insects.

The first report of toxic effect of a lectin against termites was the insecticidal activity of MuHL against *N. corniger*. When concentrations of 0.1, 0.2, 0.4 and 0.8 mg ml^{-1} of this lectin were used, it promoted mortality of termites with LC_{50} values of 0.248 mg ml^{-1} for workers and 0.199 mg ml^{-1} for soldiers (Sá et al., 2008). That study suggested that resistance of the heartwood to termite attack may be linked to termiticidal activity of MuHL. The termiticidal effect of lectins isolated from *M. urundeuva* bark and leaf (Table 1) was determined by

Napoleão et al. (2011). Both MuBL and MuLL killed workers (LC_{50} of 0.974 and 0.374 mg ml^{-1}, respectively) and soldiers (LC_{50} of 0.787 and 0.432 mg ml^{-1}, respectively).
Despite having the common property of binding to chitin, the lectins of *M. urundeuva* differently affected the survival of *N. corniger*. MuBL was less active against both castes than the other two lectins, and MuHL was more termiticidal against workers than. It has been reported that insecticidal activity of MuBL and MuLL can be due to the resistance of *M. urundeuva* lectins to proteolysis by enzymes from *N. corniger* gut, and to antibacterial action against symbiotic gut bacteria that is essential for termite survival (Napoleão et al., 2011).

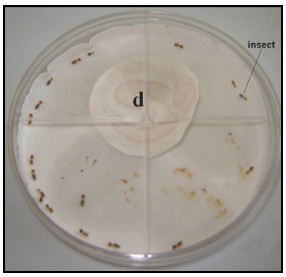


Fig. 2. Aspects of no-choice termiticidal assay used to evaluate insecticidal activity of lectins. The disk (d) of filter paper is impregnated with lectin solution.

The lectin from *B. monandra* secondary roots (BmoRoL) at concentrations of 0.025, 0.05, 0.1, 0.2 and 0.4 mg ml^{-1} also induced the mortality of *N. corniger* (Souza et al., 2011b). This lectin was more efficient against soldiers (LC_{50}: 0.014 mg ml^{-1}) than workers (LC_{50}: 0.09 mg ml^{-1}).
Termiticidal activity from *O. ficus indica* cladodes was determined using preparations (extract and OfiL) at concentrations of 0.25, 0.5, 1.0 and 1.5 mg ml^{-1} of protein (Paiva et al., 2011). The extract was termiticidal against workers at 1.5 mg ml^{-1}, though it did not interfere in survival of soldiers. OfiL was more active than cladode extracts, showing a stronger termiticidal activity against workers (LC_{50} of 0.116 mg ml^{-1}). The lectin was active against soldiers only at 1.5 mg ml^{-1}.
M. oleifera seeds were also sources of termiticidal preparations (Table 1). Bioassays used crude preparations (extracts and protein fractions) as well as purified lectins (cMoL and WSMoL) at concentrations of 0.125, 0.25, 0.5, 1.0 and 1.5 mg ml^{-1} of protein (Paiva et al., 2011). Both extracts containing cMoL and WSMoL were termiticidal on soldiers at 1.5 mg ml^{-1}, but only the protein fraction rich in WSMoL and pure WSMoL at 1.5 mg ml^{-1} interfered in the survival rate of soldiers. cMoL and WSMoL extracts (1.0 and 1.5 mg ml^{-1}), protein fraction from the WSMoL extract (1.5 mg ml^{-1}), as well the isolated cMoL and WSMoL (1.5 mg ml^{-1}) were all able to workers.
The repellent activity of MuBL, MuHL, MuLL, BmoRoL, OfiL, WSMoL and cMoL has also been investigated. Bioassays were performed in petri plates filled up with agar containing one central well at which termites were placed, and peripheral wells at which filter papers soaked with lectin were put. None of the lectins showed repellent activity *N. corniger*, since it was observed that the termites did not avoid contact with lectin-treated wells (Figure 3A).

2.4 Larvicidal activity of lectins against *Aedes aegypti*

The mosquito *A. aegypti* is native to North Africa, but it is a cosmopolitan species widely spread in tropical and subtropical regions (Forattini and Brito, 2003). Females feed more

frequently on blood than on plant sap, and have high affinity for human blood. Insect development occurs through the egg, larvae (four instars: L1, L2, L3 and L4), pupa and adult stages. Under favorable conditions of temperature, humidity and food availability, the period between the egg stage and adult emergence varies from 10 to 13 days (Forattini, 1965). *A. aegypti* is vector of human diseases of low (classic dengue) and high mortality (yellow fever and hemorrhagic dengue fever).

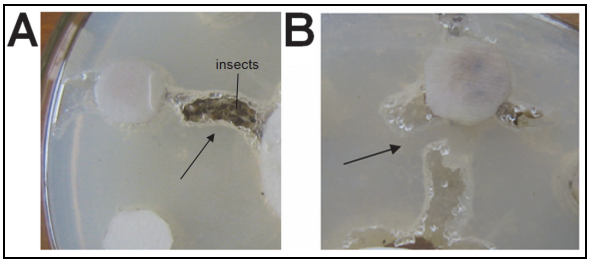


Fig. 3. Aspect of repellent activity assays. (A) non-repellent effect of lectin demonstrated by construction of tunnels in agar near a well containing lectin, and the galleries constructed in agar that remained open (arrow). (B) repellent action of methanolic extract from *M. urundeuva* heartwood detected by presence of closed gallery (arrow) constructed in agar next to peripheral wells containing the extract.

Strategies for control of *A. aegypti* immature forms includes: elimination of reproduction sites, biological control by *Bacillus thuringiensis* serovar *israelensis*, and chemical control by larvicidal oils, repellents, organophosphorous, organophosphates and pyrethroids (Luna et al., 2004; Araújo et al., 2007). The control of mosquitoes using the insecticides Temephos, Malathion and Fenitrothion is the main measure adopted by public health programs; however, *A. aegypti* larvae have developed tolerance to these compounds, and this is one of the main problems in vector control programs (Poupardin et al., 2008; Melo-Santos et al., 2010).

M. oleifera seed extracts containing WSMoL interfered in the *A. aegypti* larval development (Figure 4). First larval instar (L1) incubated with extracts prepared with one, six and fifteen seeds (SE_1, SE_6 and SE_{15}) reached the last instar (L4) after longer development times than those recorded for the negative control (distilled water). The delay in larvae development promoted by SE_6 and SE_{15} was greater than that caused by SE_1, revealing a higher concentration of active principle (Coelho et al., 2009).

WSMoL and the lectins from *M. urundeuva* bark and heartwood (MuBL and MuHL) showed larvicidal activity against fourth-stage larvae in a concentration-dependent manner (Coelho et al., 2009; Sá et al., 2009c). Figure 5 shows that these lectins promoted larvae mortality with different efficiency; the values of lectin concentration (mg ml^{-1}) required to kill 50% (LC_{50}) of larvae in 24 h were 0.125 (MuBL), 0.04 (MuHL) and 0.197 (WSMoL). The hemagglutinating activities of MuBL and MuHL were not affected by exposure to sunlight, indicating that these lectins were resistant to environmental conditions of radiation and temperature, an important characteristic to be used in *A. aegypti* control (Sá et al., 2009c).

WSMoL heated at 100 ºC for 5 h did not show hemagglutinating and larvicidal activities; these data reveal that the native protein structure is a requirement for these biological properties to remain in place. The larvae treated with WSMoL showed morphological

changes like hypertrophy of segments, increased gut volume and absence of epithelial layer that delimits the gut (Coelho et al., 2009).

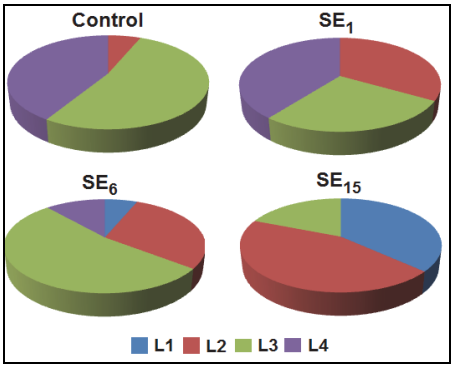


Fig. 4. Larval instar (%) of *A. aegypti* after incubation with *M. oleifera* seed extracts prepared with one, six and fifteen seeds (SE_1, SE_6 and SE_{15}, respectively) for 72 h.

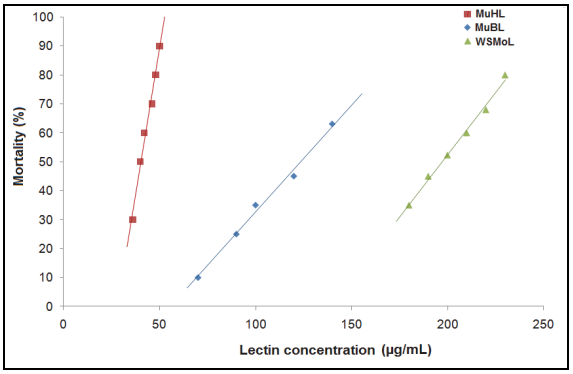


Fig. 5. Insecticidal activity of lectins from *Moringa oleifera* seeds (WSMoL) and *Myracrodruon urundeuva* bark (MuBL) and heartwood (MuHL) against *A. aegypti* fourth instar larvae (L4).

3. Secondary metabolites

Organic compounds produced by plants constitute a large and heterogeneous group known as secondary metabolites, characterized by a variety of structures and functions. They can be classified on the basis of chemical structure and composition, as nitrogen compounds (alkaloids, non-protein amino acids, amines, alcamides, cyanogenic glycosides and glucosinolates) and non-nitrogen compounds (monoterpenes, diterpenes, triterpenes, tetraterpenes, sesquiterpenes, saponins, flavonoids, steroids, coumarins).

Secondary metabolites may be found in several plant tissues. Differences in chemical properties and polarity of these molecules afford the use of different solvents for their extraction. Although aqueous extracts are usually rich in proteins, secondary metabolites can also be extracted by aqueous solutions. On the other hand, methanolic extracts generally contain large amounts of secondary metabolites with no protein content.

Natural functions and applications of secondary metabolites have been investigated employing separation techniques to isolate them from plant extracts or synthetic methods to obtaining equivalent compounds. However, many of the *in vivo* functions of secondary metabolites remain unknown. These substances do not appear to participate directly in the growth and development of plant, but many of them can be associated with survival and adaptation, including metal transporters, symbiotic agents, hormones, differential effectors and defense molecules (Demain and Fang, 2000).

The synthesis of secondary metabolites with defense role can be induced by water stress as well as seasonal variations in temperature and luminosity or infection by pathogens (Bray et al., 2000; Bulbovas et al., 2005). Experiments that simulated mechanical stimulation and damage promoted by phytopatogenous insects in *Glycine max* leaves demonstrated that secondary metabolites (mainly y-aminobutyric acid) can be accumulated in this tissue, when submitted to injuries (Ramputh and Bown, 1996).

After ingestion by herbivores, secondary metabolites can induce damage through several and different mechanisms. Alkaloids act as agonists or antagonists of neurotransmitters, and neuroreceptors or can insert themselves into DNA or induce DNA alkylation. In this way, the ingestion of alkaloids may disrupt the replication and transcription in phytophagous organisms. Non-nitrogen secondary metabolites, such as phenols, terpenoids and saponins affect herbivores through less specific mechanisms. Tannins and phenols can interact with several proteins through hydrogen bonds or ionic interactions inducing conformational changes that can lead to loss of protein activity and function. Lipophilic terpenes can affect the integrity of biomembranes. Finally, saponins have cytotoxic and antimicrobial effects by interacting with cellular membranes, inducing pore formation and causing disturbances in cell permeability (Wink, 2003).

3.1 Repellent activity of secondary metabolites from *Myracrodruon urundeuva* heartwood against *Nasutitermes corniger*

Methanolic extract from *M. urundeuva* heartwood contained secondary metabolites cinamic derivatives, flavonoids, gallic acid, luteolin, proanthocyanidins, hydrolysable tannins, and leucoanthocyanidins (Figure 6). Termiticidal and repellence bioassays revealed that the extract showed no termiticidal activity, though it induced repellent effect against *N. corniger* (Sá et al., 2009a). Insects closed the galleries constructed in agar next to peripheral wells containing the extract (Figure 3B). The presence of harmful compounds can be detected by insects through chemical receptors like olfactory or gustatory sensilla that detect chemicals with high or low-volatility, respectively (Bohbot and Vogt, 2005). Thus, termites can be repelled or attracted by a substance, depending on its chemical composition.

The studies on *M. urundeuva* heartwood indicate that two mechanisms seem to be involved in the resistance of this tissue against *N. corniger*: prevention of the arrival and attack of *N. corniger* by repellent action of secondary metabolites, and death of termites induced by MuHL, the heartwood lectin (Sá et al., 2009a).

4. Acknowledgments

The authors express their gratitude to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for research grants and fellowships (PMGP, LCBBC). We are also grateful to the Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)

for financial support. We thank Emmanuel Viana Pontual for design of the figure 1 and Felix Nonnenmacher for English editing.

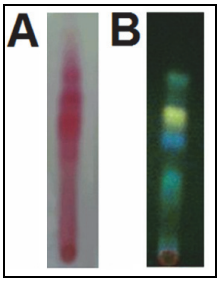


Fig. 6. Secondary metabolites from methanolic extract revealed by thin layer chromatography (TLC). (A) proanthocyanidins, hydrolysable tannins and leucoanthocyanidins. (B) kaempferol (green), quercetin (yellow) and gallic acid (blue).

5. References

Almeida-Cortez, J. S.; Cortez, P.H.M.; Franco, J.M.V. & Uzunian, A. (2007) *Caatinga - Coleção Biomas do Brasil* (1st ed), Harbra, ISBN 978-85-294-0323-1, São Paulo, Brazil.

Angus, R.B.; Dellow, J.; Winder, C. & Credland, P.F. (2011). Karyotype differences among four species of *Callosobruchus* Pic (Coleoptera: Bruchidae). *Journal of Stored Products Research*, Vol. 47, No. 2, (April 2011), pp. 76-81, ISSN 0022-474X

Araújo, A.P.; Melo-Santos, M.A.V.; Carlos, S.O.; Rios, E.M.M.M. & Regis, L. (2007). Evaluation of an experimental product based on *Bacillus thuringiensis* sorovar. *israelensis* against *Aedes aegypti* larvae (Diptera: Culicidae). *Biological Control*, Vol. 41, No. 3, (June 2007), pp. 339-347, ISSN 1049-9644

Bandyopadhyay, S.; Roy, A. & Das, S. (2001). Binding of garlic (*Allium sativum*) leaf lectin to the gut receptors of homopteran pests is correlated to its insecticidal activity. *Plant Science*, Vol. 161, No. 5, (October 2001), pp. 1025-1033, ISSN 0168-9452

Bennett, S.M. (2003). Ephestia kuehniella (Mill or Flour Moth) (can also be Mediterranean Flour Moth), in: *The PiedPiper*, 7 July 2011, Available from: <http://www.the-piedpiper.co.uk/th7e.htm>

Bohbot, J. & Vogt, R.G. (2005). Antennal expressed genes of the yellow fever mosquito (*Aedes aegypti* L.); characterization of odorant-binding protein 10 and takeout. *Insect Biochemistry and Molecular Biology*, Vol. 35, No. 9, (September 2005), pp. 961-979, ISSN 0965-1748.

Bray, E.A.; Bailey-Serres, J. & Weretilnyk, E (2000). Responses to abiotic stresses, In: *Biochemistry & Molecular Biology of Plants*, Buchanan, B.B.; Gruissen, W. & Jones L.R. (eds), pp. 1158-1203, American Society of Plant Physiologists, ISBN 0-943088-39-9, New York, US

Bulbovas, P.; Rinaldi, M.C.S.; Delitti, W.B.C. & Domingos, M. (2005). Variação sazonal em antioxidantes em folhas de plantas jovens de *Caesalpinia echinata* Lam. (pau-brasil). *Revista Brasileira de Botânica*, Vol. 28, No. 4, (December 2005), pp. 687-696, ISSN 0100-8404

Carlini, C.R. & Grossi-de-Sá, M.F. (2002). Plant toxic proteins with insecticidal properties. A review on their potential as bioinsecticides. *Toxicon*, Vol. 40, No. 11, (November 2002), pp. 1515–1539, ISSN 0041-0101

Coelho, J.S.; Santos, N.D.L.; Napoleão, T.H.; Gomes, F.S.; Ferreira, R.S.; Zingali, R.B.; Coelho, L.C.B.B.; Leite, S.P.; Navarro, D.M.A.F. & Paiva, P.M.G. (2009). Effect of *Moringa oleifera* lectin on development and mortality of *Aedes aegypti* larvae. Chemosphere, Vol. 77, No. 7, (November 2009), pp. 934-938, ISSN 0045-6535

Coelho, L.C.B.B. & Silva, M.B.R. (2000). Simple method to purify milligram quantities of the galactose-specific lectin from the leaves of *Bauhinia monandra*. *Phytochemical Analysis*, Vol. 11, No. 5, pp. 295-300, ISSN 0958-0344

Coelho, M.B.; Marangoni, S. & Macedo, M.L.R. (2007). Insecticidal action of *Annona coriacea* lectin against the flour moth *Anagasta kuehniella* and the rice moth *Corcyra cephalonica* (Lepidoptera: Pyralidae). *Comparative Biochemistry and Physiology Part C, Toxicology and Pharmacology* Vol. 146, No. 3, (September 2007), pp. 406-414, ISSN 1532-0456

Credland, P.F. & Dendy, J. (1992). Intraspecific variation in binomic characters of the Mexican bean weevil, *Zabrotes subfasciatus*. *Entomologia Experimentalis et Applicata*, Vol. 65, No. 1, (October 1992), pp. 39-47, ISSN 1570-7458

Demain, A.L. & Fang A. (2000). The natural functions of secondary metabolites. *Advances in Biochemical Engineering Biotechnology*. Vol. 69, (Year 2000), pp. 1–39, ISSN 0724-6145

Edvardsson, M. & Tregenza, T. (2005). Why do male *Callosobruchus maculatus* harm their mates? *Behavioral Ecology*, Vol. 16, No. 4, (July/August 2005), pp. 788-793, ISSN 1045-2249

Edwards, P.J.; Wratten, S.D. (1980). *Ecology of insect-plant interactions*. Edward Arnold, ISBN 9780713128031, London, England

Eisemann, C.H.; Donaldson, R.A.; Pearson, R.D.; Cadogan, L.C.; Vacuolo, T. & Tellam, R.L. (1994). Larvicidal activity of lectins on *Lucilia cuprina*: mechanism of action. *Entomologia Experimentalis et Applicata*, Vol. 72, No. 1, (July 1994), pp. 1–10, ISSN 1570-7458

Ferreira, R.S.; Napoleão, T.H.; Santos, A.F.S.; Sá, R.A.; Carneiro-da-Cunha, M.G.; Morais, M.M.C.; Silva-Lucca, R.A.; Oliva, M.L.V.; Coelho, L.C.B.B. & Paiva, P.M.G. (2011) Coagulant and antibacterial activities of the water-soluble seed lectin from *Moringa oleifera*. *Letters in Applied Microbiology*, Vol. 53, No. 2, (August 2011), pp. 186-192 ISSN 1472-765X

Fitches, E.; Woodhouse, S.D.; Edwards, J.P. & Gatehouse, J.A. (2001). *In vitro* and *in vivo* binding of snowdrop (*Galanthus nivalis* agglutinin; GNA) and jackbean (*Canavalia ensiformis*; Con A) lectins within tomato moth (*Lacanobia oleracea*) larvae; mechanisms of insecticidal action. *Journal of Insect Physiology*, Vol. 47, No. 7, (July 2011), pp. 777-787, ISSN 0022-1910

Fitches, E.; Philip, J.; Hinchliffe, G.; Vercruysse, L.; Chougule, N. & Gatehouse, J.A. (2008). An evaluation of garlic lectin as an alternative carrier domain for insecticidal fusion proteins. *Insect Science*, Vol. 15, No. 6, (December 2008), pp. 483-495, ISSN 1744-7917

Forattini, O.P. (1965). *Entomologia Médica, volume 2* (2nd ed), Editora da Universidade de São Paulo, São Paulo, Brazil.

Forattini, O.P.; Brito, M. (2003). Household water resevoirs and control of *Aedes aegypti*. *Revista de Saúde Pública*, Vol. 37, No. 5, (October 2003), pp. 676-677, ISSN 0034-8910

Galati, E.M.; Monforte, M.T.; Tripodo, M.N.; D'Aquino, A. & Mondello, M.R. (2001). Antiulcer activity of *Opuntia ficus indica* (L) Mill. (Cactaceae): ultrastructural study. *Journal of Ethnopharmacology*, Vol. 76, No. 1, (June 2001), pp. 1-9, ISSN 0378-8741

Galati, E.M.; c Miceli, N. & Monforte, M.T. (2002). Biological effect of *Opuntia ficus indica* (L) Mill. (Cactaceae) waste matter. Note I: diuretic activity. *Journal of Ethnopharmacology*, Vol. 79, No. 1, (February 2002), pp. 17-21, ISSN 0378-8741

Gallo, D.; Nakano, O.; Neto, S.S.; Carvalho, R.P.L.; Batista, G.C.; Filho, E.B.; Parra, J.R.P.; Zucchi, R.A.; Alves, S.B.; Vendramim, J.D.; Marchini, L.C.; Lopes, J.R.S. & Omoto, C. (2002). *Entomologia agrícola* (1st ed), FEALQ, ISBN 85-7133-011-5, Piracicaba, Brazil.

Gassenschmidt, U.; Jany, K.D.; Tauscher, B. & Nierbergall, H. (1995). Isolation and characterization of a flocculating protein from *Moringa oleifera* Lam. *Biochimica et Biophysica Acta*, Vol. 1243, No. 3, (13 April 1995), pp. 477-481, ISSN 0304-4165

Ghebremichael, K.A.; Gunaratna, K.R.; Henriksson, H.; Brumer, H.; Dalhammar, G. (2005). A simple purification and activity assay of the coagulant protein from *Moringa oleifera* seed. *Water Research*, Vol. 39, No. 11, (June 2005), pp. 2338-2344, ISSN 0043-1354

Harper, M.S.; Hopkins, T.L. & Czapla, T.H. (1998). Effect of wheat germ agglutinin on the formation and structure of the peritrophic membrane in European corn borer (*Ostrinia nubilalis*) larvae. *Tissue and Cell*, Vol. 30, No. 2, (April 1998), pp. 166–176, ISSN 0040-8166

Hegedus, D.; Erlandson, M.; Gillott, C.; Toprak, U. (2009). New Insights into Peritrophic Matrix Synthesis, Architecture, and Function. *Annual Review of Entomology*, Vol. 54, (Year 2009), pp. 285-302, ISSN 0066-4170

Hinata, K. & Nishio, T. (1981). Con A-Peroxidase method: an improved procedure for staining *S*-glycoproteins in cellulose-acetate electrofocusing in crucifers. *Theoretical and Applied Genetics*, Vol. 60, No. 5, (September 1981), pp. 281-283, ISSN 0040-5752

Hogervorst, P.A.M.; Ferry, N.; Gatehouse, A.M.R.; Wäckers, F.L. & Romeis, J. (2006). Direct effects of snowdrop lectin (GNA) on larvae of three aphid predators and fate of GNA after ingestion. *Journal of Insect Physiology*, Vol. 52, No. 6, (June 2006), pp. 614-624, ISSN 0022-1910

Isidro, R.; Sales, F.J.M.; Cavada, B.S.; Grangeiro, T.B. & Moreira, R.A. (2001) Ação de lectina de sementes de *Canavalia brasiliensis* Mart. sobre o comportamento da saúva do Nordeste (*Atta opaciceps* Borgmeier, 1939). *Revista de la Facultad de Agronomía (Maracay)*, Vol. 27, No. 2, p. 77-86, ISSN 1856-5573

Judd, W.S.; Campbell, C.S.; Kellog, E.A.; Stevens, P.F. & Donoghue, M.J. (2007). *Plant Systematics: A Phylogenetic Approach* (3rd ed), Sinauer Associates Inc., ISBN 978-0878934072, Sunderland, US.

Kaur, M.; Singh, K.; Rup, P.J.; Saxena, A.K.; Khan, R.H.; Ashraf, M.T.; Kamboj, S.S.; Singh, J. (2006). A tuber lectin from *Arisaema helleborifolium* Schott with anti-insect activity against melon fruit fly, *Bactrocera cucurbitae* (Coquillett) and anti-cancer effect on

human cancer cell. *Archives of Biochemistry and Biophysics*, Vol. 445, No. 1, (1 January 2006), pp. 156-165, ISSN 0003-9861

Kawsar, S.M.; Fujii, Y.; Matsumoto, R.; Ichikawa, T.; Tateno, H.; Hirabayashi, J.; Yasumitsu, H.; Dogasaki, C.; Hosono, M.; Nitta, K.; Hamako, J.; Matsui, T. & Ozeki, Y. (2008). Isolation, purification, characterization and glycan-binding profile of a D-galactoside specific lectin from the marine sponge, *Halichondria okadai*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, Vol. 150, No. 4, (August 2008), pp. 349-357, ISSN 1096-4959

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, Vol. 227, (August 1970), pp. 680-685, ISSN 0028-0836

Leite, E.J. (2002). State of knowledge on *Myracrodruon urundeuva* Fr. Allemão (Anacardiaceae) for genetic conservation in Brazil. *Perspectives in Plant Ecology, Evolution and Systematics*, Vol. 5, No. 3, pp. 193-206, ISSN 1433-8319

Li, H.M.; Sun, L.; Mittapalli, O.; Muir, W.M.; Xie, J.; Wu, J.; Schemerhorn, B.J.; Sun, W.; Pittendrigh, B.R. &, Murdock, L.L. (2009). Transcriptional signatures in response to wheat germ agglutinin and starvation in *Drosophila melanogaster* larval midgut. *Insect Molecular Biology*, Vol. 18, No. 1, (February 2009), pp. 21–31, ISSN 1365-2583

Loganathan, M.; Jayas, D.S.; Fields, P.G. & White, N.D.G. (2011). Low and high temperatures for the control of cowpea beetle, *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) in chickpeas. *Journal of Stored Products Research*, Vol. 47, No. 3, (July 2011), pp. 244-248, ISSN 0022-474X

Luna, J.E.D.; Martins, M.F.; Anjos, A.T.; Kuwabaro, E.F. & Navarro-Silva, M.A. (2004). Susceptibilidade de *Aedes aegypti* aos inseticidas temephos e cipermetrina, Brasil. *Revista de Saúde Pública*, Vol. 38, No. 6, (December 2004), pp. 842-843, ISSN 0034-8910

Macedo, M.L.R.; Damico, D.C.S.; Freire, M.G.M.; Toyama, M.H.; Marangoni, S. & Novello, J.C. (2003). Purification and characterization of an *N*-acetylglucosamine-binding lectin from *Koelreuteria paniculata* seeds and its effect on the larval development of *Callosobruchus maculatus* (Coleoptera: Bruchidae) and *Anagasta kuehniella* (Lepidoptera: Pyralidae). *Journal of Agricultural and Food Chemistry*, Vol. 51, No. 10, (April 2003), pp. 2980-2986, ISSN 0021-8561

Macedo, M.L.R.; Castro, M.M. & Freire, M.G.M. (2004). Mechanisms of the insecticidal action of TEL (*Talisia esculenta* lectin) against *Callosobruchus maculatus* (Coleoptera: Bruchidae). *Archives of Insect Biochemistry and Physiology*, Vol. 56, No. 2, (June 2004), pp. 84–96, ISSN 1520-6327

Macedo, M.L.R.; Freire, M.G.M.; Silva, M.B.R. & Coelho, L.C.B.B. (2007). Insecticidal action of *Bauhinia monandra* leaf lectin (BmoLL) against *Anagasta kuehniella* (Lepidoptera: Pyralidae), *Zabrotes subfasciatus* and *Callosobruchus maculatus* (Coleoptera: Bruchidae). *Comparative Biochemistry and Physiology Part A, Molecular and Integrative Physiology*, Vol. 146, No. 4, (April 2007), pp. 486-498, ISSN 1095-6433

Mainieri, C.; Chimelo, J.P. (1989). *Fichas de características de madeiras brasileiras*. IPT, ISBN 978-850-9000-46-2 São Paulo, Brazil.

Melo-Santos, M.A.V., Varjal-Melo, J.J.M., Araújo, A.P., Gomes, T.C.S., Paiva, M.H.S., Regis, L.N., Furtado, A.F., Magalhães, T., Macoris, M.L.G., Andrighetti, M.T.M. & Ayres,

C.F.J. (2010). Resistance to the organophosphate temephos: mechanisms, evolution and reversion in an *Aedes aegypti* laboratory strain from Brazil. *Acta Tropica*, Vol. 113, No. 2, (February 2010), pp. 180–189, ISSN 0001-706X

Monath, T.P. (2008) Treatment of yellow fever. *Antiviral Research*, Vol. 78, No. 1, (April 2008), pp. 116-124, ISSN 0166-3542

Moreno, F.B.; Oliveira, T. M.; Martil, D.E.; Viçoti, M.M.; Bezerra, G.A.; Abrego, J.R.; Cavada, B.S. & Azevedo Jr., W.F. (2008). Identification of a new quaternary association for legume lectins. *Journal of Structural Biology*, Vol. 161, No. 2, (February 2008), pp. 133-143, ISSN 1047-8477

Murdock, L.L.; Huesing, J.E.; Nielsen, S.S.; Pratt, R.C. & Shade, R.E. (1990). Biological effects of plant lectins on the cowpea weevil. *Phytochemistry*, Vol. 29, No. 1, pp. 85-89, ISSN 0031-9422

Napoleão, T.H.; Gomes, F.S.; Lima, T.A.; Santos, N.D.L.; Sá, R.A.; Albuquerque, A.C.; Coelho, L.C.B.B. & Paiva, P.M.G. (2011). Termiticidal activity of lectins from *Myracrodruon urundeuva* against *Nasutitermes corniger* and its mechanisms. *International Biodeterioration & Biodegradation*, Vol. 65, No. 1, (January 2011), pp. 52-59, ISSN 0964-8305

Nasi, A.; Picariello, G.; Ferranti, P. (2009). Proteomic approaches to study structure, functions and toxicity of legume seeds lectins. Perspectives for the assessment of food quality and safety. *Journal of Proteomics*, Vol. 72, No. 3, (13 April 2009), pp. 527-538, ISSN 1874-3919

Ndabigengesere, A.; Narasiah, K.S. & Talbot, B.G. (1995). Active agents and mechanism of coagulation of turbid waters using *Moringa oleifera*. *Water Research*, Vol. 29, No. 2, (February 1995), pp. 703-710, ISSN 0043-1354

Okuda, T., Baes, A.U., Nishijima, W. & Okada, M. (2001). Isolation and characterization of coagulant extracted from *Moringa oleifera* seed by salt solution. *Water Research*, Vol. 35, No. 2, (February 2001), pp. 405-410, ISSN 0043-1354

Oliveira, C.F.R.; Luz, L.A.; Paiva, P.M.G.; Coelho, L.C.B.B.; Marangoni, S. & Macedo, M.L.R. (2011). Evaluation of seed coagulant *Moringa oleifera* lectin (cMoL) as a bioinsecticidal tool with potential for the control of insects. *Process Biochemistry*, Vol. 46, No. 2, (February 2011), pp. 498-504, ISSN 1359-5113

Paes, J.B.; Morais, V.M. & Lima, CR (2002). Resistência das madeiras de aroeira (*Myracrodruon urundeuva*), cássia (*Senna siamea*) e ipê (*Tabebuia impetiginosa*) a fungos e cupins xilófagos em condições de laboratório. *Floresta e Ambiente*, Vol. 9, No. 1, (Year 2002), pp. 135–144, ISSN 1415-0980

Paes, J.B.; Melo, R.R.; Lima, C.R. & Oliveira, E. (2007). Resistência natural de sete madeiras ao cupim subterrâneo (*Nasutitermes corniger* Motsch.) em ensaio de preferência alimentar. *Revista Brasileira de Ciências Agrárias*, Vol. 2, No. 1, (January-March 2007), pp. 57-62, ISSN 1981-0997

Paiva, P.M.G.; Santana, G.M.S.; Souza, I.F.A.C.; Albuquerque, L.P.; Agra-Neto, A.C.; Albuquerque, A.C.; Luz, L.A.; Napoleão, T.H.; Coelho, L.C.B.B. (2011). Effect of lectins from *Opuntia ficus indica* cladodes and *Moringa oleifera* seeds on survival of *Nasutitermes corniger*. *International Biodeterioration & Biodegradation*, doi:10.1016/ j.ibiod.2011.05.008.

Peumans, W.J.; Van Damme, E.J.M. (1995). Lectins as plant defense proteins. *Plant Physiology*, Vol. 109, No. 2, (October 1995), pp. 347-352, ISSN 0032-0889

Pharmacia Fine Chemicals (1980). *Polyacrylamide Electrophoresis, Laboratory Techniques*, Pharmacia [Pamphlet], ASIN B002O1CL3C, Uppsala, Sweden.

Poupardin, R.; Reynaud, S.; Strode, C.; Ranson, H.; Vontas, J.; David, J.P. (2008). Cross-induction of detoxification genes by environmental xenobiotics and insecticides in the mosquito *Aedes aegypti*: impact on larval tolerance to chemical insecticides. *Insect Biochemistry and Molecular Biology*, Vol. 38, No. 5, (May 2008), pp. 540-551, ISSN 0965-1748

Powell, K.S.; Gatehouse, A.M.R.; Hilder, V.A.; Gatehouse, J.A. (1995). Antifeedant effects of plant-lectins and an enzyme on the adult stage of the rice brown planthopper, *Nilaparvata lugens*. *Entomologia Experimentalis et Applicata*, Vol. 75, No. 1, (April 1995), pp. 51-59, ISSN 1570-7458

Ramputh, A-I. & Brown, A.W. (1996). Rapid γ-aminobutyric acid synthesis and the lnhibition of the growth and development of oblique-banded leaf-roller larvae. *Plant Physiology*, Vol. 111, No. 4, (August 1996), pp. 1349-1352, ISSN 0032-0889

Rao, V.S.; Viana, G.S.B.; Menezes, A.M.S.; Gadelha, M.G.T. (1987). Studies on the anti-ulcerogenic activity of *Astronium urundeuva*. *Brazilian Journal of Medical and Biological Research*, Vol. 20, No. 6, pp. 253–256, ISSN 0100-879X

Reisfeld, R.A., Lewis, U.J. & Williams, D.E. (1962). Disk eletrophoresis of basic protein and peptides on polyacrylamide gels. *Nature*, Vol. 195 (July 1962), pp. 281-283, ISSN 0028-0836

Rolim, L.A.D.M.M.; Macedo, M.F.S.; Sisenando, H.A.; Napoleão, T.H.; Felzenswalb, I.; Aiub, C.A.F.; Coelho, L.C.B.B.; Medeiros, S.R.B. & Paiva, P.M.G. (2011). Genotoxicity evaluation of *Moringa oleifera* seed extract and lectin. *Journal of Food Science*, Vol. 76, No. 2, (March 2011), pp. T53-T58, ISSN 1750-3841

Sá, R.A.; Argolo, A.C.C.; Napoleão, T.H.; Gomes, F.S.; Santos, N.D.L.; Melo, C.M.L.; Albuquerque, A.C.; Xavier, H.S.; Coelho, L.C.B.B.; Bieber, L.W. & Paiva, P.M.G. (2009a). Antioxidant, *Fusarium* growth inhibition and *Nasutitermes corniger* repellent activities of secondary metabolites from *Myracrodruon urundeuva* heartwood. *International Biodeterioration & Biodegradation*, Vol. 63, No. 4, (June 2009), pp. 470-477, ISSN 0964-8305

Sá, R.A.; Gomes, F.S.; Napoleão, T.H.; Santos, N.D.L.; Melo, C.M.L.; Gusmão, N.B.; Coelho, L.C.B.B.; Paiva, P.M.G. & Bieber, L.W. (2009b). Antibacterial and antifungal activities of *Myracrodruon urundeuva* heartwood. *Wood Science and Technology*, Vol. 43, No. 1-2, (February 2009), pp. 85-95, ISSN 1432-5225

Sá, R.A.; Napoleão, T.H.; Santos, N.D.L.; Gomes, F.S.; Albuquerque, A.C.; Xavier, H.S.; Coelho, L.C.B.B.; Bieber, L.W. & Paiva, P.M.G. (2008). Induction of mortality on *Nasutitermes corniger* (Isoptera, Termitidae) by *Myracrodruon urundeuva* heartwood lectin. *International Biodeterioration & Biodegradation*, Vol. 62, No. 4, (December 2008), pp. 460-464, ISSN 0964-8305

Sá, R.A.; Santos, N.D.L.; da Silva, C.S.B.; Napoleão, T.H.; Gomes, F.S.; Cavada, B.S.; Coelho, L.C.B.B.; Navarro, D.M.A.F.; Bieber, L.W. & Paiva, P.M.G. (2009c). Larvicidal activity of lectins from *Myracrodruon urundeuva* on *Aedes aegypti*. *Comparative*

Biochemistry and Physiology Part C, Toxicology and Pharmacology, Vol. 149, No. 3, (April 2009), pp. 300-306, ISSN 1532-0456

Santana, G.M.S.; Albuquerque, L.P.; Simões, D.A.; Gusmão, N.B.; Coelho, L.C.B.B. & Paiva, P.M.G. 2009. Isolation of lectin from *Opuntia ficus indica* cladodes. *Acta Horticulturae*, Vol. 811, (February 2009), pp. 281-286, ISSN 0567-7572

Santana, M.A.; Santos, A.M.C.; Oliveira, M.E.; de Oliveira, J.S.; Baba, E.H.; Santoro, M.M.; de Andrade, M.H.G. (2008). A novel and efficient and low-cost methodology for purification of *Macrotyloma axillare* (Leguminosae) seed lectin. *International Journal of Biological Macromolecules*, Vol. 43, No. 4, (November 2008), pp. 352-358, ISSN 0141-8130

Santos, A.F.S.; Argolo, A.C.C.; Coelho, L.C.B.B.; Paiva, P.M.G. (2005). Detection of water soluble lectin and antioxidant component from *Moringa oleifera* seeds. *Water Research*, Vol. 39, No. 6, (March 2005), pp. 975-980, ISSN 0043-1354

Santos, A.F.S.; Carneiro-da-Cunha, M.G.; Teixeira, J.A.; Paiva, P.M.G.; Coelho, L.C.B.B. & Nogueira, R.M.O.B. (2011a). Interaction of *Moringa oleifera* seed lectin with humic acid. *Chemical Papers*, Vol. 65, No. 4, (August 2011), pp. 406-411, ISSN 1336-9075

Santos, A.F.S.; Luz, L.A.; Argolo, A.C.C.; Teixeira, J.A.; Paiva, P.M.G. & Coelho, L.C.B.B. (2009). Isolation of a seed coagulant *Moringa oleifera* lectin. *Process Biochemistry*, Vol. 44, No. 4, (April 2009), pp. 504–508, ISSN 1359-5113

Santos, A.F.S.; Paiva, P.M.G.; Teixeira, J.A.C.; Brito, A.G.; Coelho, L.C.B.B. & Nogueira, R. (2011b). Coagulant properties of *Moringa oleifera* protein preparations: application to humic acids removal. *Environmental Technology*, doi:0.1080/ 09593330.2010.550323, ISSN 1479-487X

Sari, L.T.; Ribeiro-Costa, C.S. & Pereira, P.R.V.S. (2003). Biological aspects of *Zabrotes subfasciatus* (Bohemann, 1833) (Coleoptera, Bruchidae) on *Phaseolus vulgaris* L., cv. Carioca (Fabaceae), under laboratory conditions. *Revista Brasileira de Entomologia*, Vol. 47, No. 4, (December 2003), pp. 621-624, ISSN 0085-5626

Scheffrahn, R.H.; Cabrera, B.J.; Kern Jr., W.J. & Su, N.Y. (2002). *Nasutitermes costalis* (Isoptera: Termitidae) in Florida: first record of a non-endemic establishment by a higher termite. *Florida Entomologist*, Vol. 85, No. 1, (March 2002), pp. 273-275, ISSN 0015-4040

Scheffrahn, R.H.; Krecek, J.; Szalanski, A.L. & Austin, J.W. (2005). Synonymy of neotropical arboreal termites *Nasutitermes corniger* and *N. costalis* (Isoptera: Termitidae: Nasutitermitinae) with evidence from morphology, genetics, and biogeography. *Annals of the Entomological Society of America*, Vol. 98, No. 3, (May 2005), pp. 273-281, ISSN 0013-8746

Sharon, N. & Lis, H. (2001). The structural basis for carbohydrate recognition by lectins. *Advances in Experimental Medicine and Biology*, Vol. 491, pp. 1-16.

Sharon, N. & Lis, H. (2007). *Lectins* (2nd ed), Springer, ISBN 978-1-4020-6605-4, Dordrecht, The Netherlands.

Silva, M.D.C.; Sá, R.A.; Napoleão, T.H.; Gomes, F.S.; Santos, N.D.L.; Albuquerque, A.C.; Xavier, H.S.; Paiva, P.M.G.; Correia, M.T.S. & Coelho, L.C.B.B. (2009). Purified *Cladonia verticillaris* lichen lectin: Insecticidal activity on *Nasutitermes corniger*

(Isoptera: Termitidae). *International Biodeterioration & Biodegradation*, Vol. 63, No. 3, (April 2009), pp. 334-340, ISSN 0964-8305

Sisenando, H.A.A.A.C.N.; Macedo, M.F.S.; Saturnini, A.C.R.D.; Coelho, L.C.B.B. & Medeiros, S.R.B. (2009). Evaluation of the genotoxic potential of *Bauhinia monandra* leaf lectin (BmoLL). *Food and Chemical Toxicology*, Vol. 47, No. 2, (February 2009), pp. 303-308, ISSN 0278-6915

Souza, A.J.; Santos, P.O.; Pinto, M.S.T.; Wermelinger, T.T.; Ribeiro, E.S.; Souza, S.C.; Deus, M.F.; Souza, M.C.; Xavier-Filho, J.; Fernandes, K.V.S. & Oliveira, A.E.A. (2011a). Natural seed coats provide protection against penetration by *Callosobruchus maculatus* (Coleoptera: Bruchidae) larvae. *Crop Protection*, Vol. 30, No. 6, (June 2011), pp. 651-657, ISSN 0261-2194

Souza, J.D.; Silva, M.B.R.; Argolo, A.C.C.; Napoleão, T.H.; Sá, R.A.; Correia, M.T.S.; Paiva, P.M.G.; Silva, M.D.C. & Coelho, L.C.B.B. (2011b). A new *Bauhinia monandra* galactose-specific lectin purified in milligram quantities from secondary roots with antifungal and termiticidal activities. *International Biodeterioration and Biodegradation*, Vol. 65, No. 5, (August 2011), pp. 696-702, ISSN 0964-8305

Tegegne, F.; Kijora, C. & Peters, K. (2007). Study on the optimal level of cactus pear (*Opuntia ficus-indica*) supplementation to sheep and its contribution as source of water, *Small Ruminant Research*, Vol. 72, No. 2-3, (October 2007), pp. 157–164, ISSN 0921-4488

Tellam, R.L.; Wijffels, G. & Wiladsen, P. (1999). Peritrophic matrix proteins. *Insect Biochemistry and Molecular Biology*, Vol. 29, No. 2, (February 1999), pp, 87-101, ISSN 0965-1748.

Tounsi, S.; Dammak, M.; Rebaî, A. & Jaoua, S. (2005). Response of larval *Ephestia kuehniella* (Lepidoptera: Pyralidae) to individual *Bacillus thuringiensis kurstaki* toxins and toxin mixtures. *Biological Control*, Vol. 35, No. 1, (October 2005), pp. 27-31, ISSN 1049-9644

Trombetta, D.; Puglia, C.; Perri, D.; Licata, A.; Pergolizzi, S.; Lauriano, E.R.; De Pasquale, A.; Saija, A. & Bonina, F.P. (2006). Effect of polysaccharides from *Opuntia ficus indica* (L.) cladodes on the healing of dermal wounds in the rat. *Phytomedicine*, Vol. 13, No. 5, (9 May 2006), pp. 352-358, ISSN 0944-7113

Vandenborre, G.; Smagghe, G. & Van Damme, E.J.M. (2011). Plant lectins as defense proteins against phytophagous insects. *Phytochemistry*, Vol. 72, No. 13 (September 2011), pp. 1538-1550, ISSN 0031-9422

Wink, M. (2003). Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry*, Vol. 64, No. 1, (September 2003), pp. 3-19, ISSN 0031-9422

Wu, A.M.; Song, S.C.; Tsai, M.S., Herp, A. (2001) A guide to the carbohydrate specificities of applied lectins-2 (updated in 2000). *Advances in Experimental Medicine and Biology*, Vol. 491, pp. 551–585, ISSN 0065-2598

Yu, L.G.; Fernig, D.G.; White, M.R.H.; Spiller, D.G.; Appleton, P.; Evans, R.C.; Grierson, I.; Smith, J.A.; Davies, H.; Gerasimenko, O.V.; Petersen, O.H.; Milton, J.D. & Rhodes, J.M. (1999). Edible mushroom (*Agaricus bisporus*) lectin, which reversibly inhibits epithelial cell proliferation, blocks nuclear localization sequence-dependent nuclear protein import. *The Journal of Biological Chemistry*, Vol. 274, No. 8, (February 1999), pp. 4890-4899, ISSN 0021-9258

Zhu-Salzman, K.; Salzman, R.A. (2001). Functional mechanics of the plant defensive *Griffonia simplicifolia* lectin II: resistance to proteolysis is independent of glycoconjugate binding in the insect gut. *Journal of Economic Entomology*, Vol. 94, No. 5, (October 2001), pp. 1280–1284, ISSN 0022-0493

Zhu-Salzman, K.; Shade, R.E.; Koiwa, H.; Salzman, R.A.; Narasimhan, M.; Bressan, R.A.; Hasegawa, P.M. & Murdock, L.L. (1998). Carbohydrate binding and resistance to proteolysis control insecticidal activity of *Griffonia simplicifolia* lectin II. *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 95, (December 1998), pp. 15123–15128, ISSN 0027-8424

Part 5

Advances in Pest Control

26

Mosquito Control Aerosols' Efficacy Based on Pyrethroids Constituents

Dylo Pemba and Chifundo Kadangwe
Biology Department Chancellor College
University of Malawi
Malawi

1. Introduction

Mosquitoes are found all over the world except in the Antarctica. Mosquitoes are two winged insects that belong to the insect order Dipteran. Members of the genera *Aede*s, *Culex* and *Anopheles* are well known and responsible for bites in humans. Only Female mosquitoes bite. This is because they require blood to assist in vitellogenesis. Feeding behaviour of mosquitoes is either classified as zoophilic or anthropophilic. Mosquito species with zoophilic behaviour prefer feeding on animals. Mosquitoes with anthropophilic behaviour prefer feeding on human blood. (Fradin, 1998). Mosquitoes are vectors of many life threatening diseases in humans. They also transmit disease in animals such as dogs and horses. Diseases transmitted include malaria, dog heart worm, West Nile virus, and Eastern Equine Encephalitis (Pemba, 2008).

Malaria is Africa's major cause of mortality in those younger than 5 years of age and constitutes 10% of the continent's overall disease burden (Farenhorst et al, 2008). To reduce the ability of mosquitoes to transmit diseases requires well planned strategies and several methods have been developed (Pemba, 2008). the main stay of mosquito control are chemicals also known as insecticides which could be in form of insect growth regulators, biopesticides, genetically engineered biopesticides, repellents and attractants (WHO, 1996).

Insecticide resistance is one of the biggest challenges in pest control (Diabate, 2002). Insecticide resistance is the second biggest challenge in vector control besides resource availability. Resistance has drastically affected mosquito control programs like insecticide-treated nets in Africa , indoor residual sprayings (asia)and fogging sprays in the Caribbean (Bonnet et al, 2009).

Some factors contributing to resistance are genetic changes and metabolic changes in the vector/pest body. Genetic resistance comes about when the site usually targeted by an insecticide changes as a result of gene shuffling and metabolic resistance is a result of increased detoxification of a particular insecticide In the Caribbean, where dengue is one of the major health problems; the vector *Aedes aegypti* has wide insecticide resistance developed. To establish efficacy of pyrethroid and organophosphate ultra-low volume space sprays studies were conducted in Martinique where *Aedes aegypti* has been shown to be resistant to conventional insecticides. Wild type population showed high levels of resistance to deltamethrin, organophosphate (naled), and pyrethrum. Simulated-field trials showed

that this resistance can strongly reduce the knock-down effect and mortality of deltamethrin and synergized pyrethrins. This finding has important implications for dengue vector control and emphasizes the need to develop innovative strategies to maintain effective control of resistant in *Ae. aegypti* populations (Marcombe et al,2009).

Studies in aerosol efficacy have focused on the atomisation and charge properties of the particles. At low concentrations of 1.57 g kg−1 of bioallethrin and 0.29 g kg−1 of bioresmethrin, charged aerosols achieve a significant reduction in KDT_{50} by up to 50%. In one study by Khadri, .bioefficacy of three pyrethroid aerosols on mosquitoes was tested in simulated room conditions. Each aerosol product was tested based on the insecticide manufacturers' recommended dosages. All the aerosols induced complete or very high mortality. Insecticide droplet analysis indicated variable uniformity of the droplets was produced. The aerosol insecticides were effective against mosquitoes provided they were used in accordance with the manufacturers' recommendations (Khadri et al 2009).

Resistance is overcome by changing insecticide, as long as the insecticides do not work the same way(mode of action), are not similar in formulation so as not similarly detoxified. As a way of resistance management rotational use of insecticides or combinations are adopted. One method that relies very much on insecticide combination for improved efficacy as well as resistance management is in insecticide aerosol formulations. The back bone of space sprays formulation are pyrethroids.

Pyrethroids are mostly used because of their low volatility, high insecticidal potency, and low toxicity to mammals under normal conditions of use (Cakir et al, 2008). Pyrethroids are synthetic chemicals very similar in structure to naturally occurring pyrethrins , but are often more toxic to insects, as well as to mammals, and last longer in the environment than pyrethrins. More than 1,000 synthetic pyrethroids have been developed, but only a few of them are currently used.

Pyrethroids are synthetic esters derived from the naturally-occurring pyrethrins. One exception to the axiom that all pyrethroids are esters of carboxylic acids is noteworthy. There is a group of oxime ethers that exhibits insecticidal activity similar in nature to the pyrethrins and pyrethroid esters (Davies 1985). Little data exist regarding these compounds, and no commercial products have been produced. Commercially available pyrethroids include allethrin, bifenthrin, bioresmethrin, cyfluthrin, cyhalothrin, cypermethrin, deltamethrin, esfenvalerate (fenvalerate), flucythrinate, flumethrin, fluvalinate, fenpropathrin, permethrin, phenothrin, resmethrin, tefluthrin, tetramethrin, and tralomethrin.

Most commercial pyrethroids are not one single molecule; rather, they are several molecules with the same chemical formula that have their atoms joined together in the same sequence, but have a different arrangement of the atoms in space. Such compounds are called stereoisomers . If the stereoisomers are not mirror images of one another, they are called diastereomers and have different physical properties like boiling point, melting point, and solubility. If they are non-superimposable mirror images of each other, they are called enantiomers and properties like boiling point, melting point, and solubility are identical. However, both diastereomers and enantiomers can have different insecticidal properties and different toxicities. Some pyrethroids are composed of as many as eight different stereoisomers. Pyrethrins and pyrethroids are often combined commercially with other chemicals called synergists, which enhance the insecticidal activity of the pyrethrins and pyrethroids. The synergists prevent enzymes from breaking down the pyrethrins and

pyrethroids, thus increasing their toxicity. Studies have shown that detoxication of xenobiotics such as synthetic pyrethroids is catalyzed by monooxygenases (MO) and nonspecific esterases (NE). Piperonyl butoxide (PBO) and MGK-264 are known inhibitors of insect detoxication systems. Both synergists are used in various insecticide compositions, mainly in aerosol cans. Best synergism occur in a mixture of PBO or MGK-264 with pyrethrins. Metabolic resistance to insecticides easily overcome by use of synergists. Several registered insecticide compositions include the synergist PBO which is to improve the Efficacy of the insecticide (Eremina2002). The great success of pyrethroids is also related to their strong efficacy at low dose, fast killing effect and relative low cost of production. (Bonnet et al, 2009). Household synthetic pyrethoids used as space sprays contain isomers such as permethrin, d-tetramethrin, esbiothrin and deltamethrin either alone or in combination with other synthetic pyrethroids (Rapeeporn et al, 2005). To come up with a recommendation on which aerosol combination is most effective has not been a primary focus of scientists. This has made it difficult for buyers to make a decision when buying since so many aerosols are on the market.

2. Aerosol sprays

What are aerosols:. Aerosol spray is a type of dispensing system which creates an aerosol mist of liquid particles. This is used with a can or bottle that contains a liquid under pressure intended to be delivered to their biological targets. The liquid under pressure comes out as a mist. The pressure inside the container remain constant as the payload is delivered by an evaporating liquid to gas propellant. Outside the can, the droplets of propellant evaporate rapidly, leaving the needed fine particles or droplets in this case insecticide- floating in the air.

Aerosol insecticides are easier to use and deliver than other forms of insect killers as such does not need professionals to use. They also work faster as there is no need to wait for an insect to approach as with baited mode of insecticide delivery. The insecticide floats around like a gas filling the volume/space thus reaching and landing on everything including the target insect, that could be hiding in even tight spaces. Aerosol insecticides are available in containers designed to prevent waste. Some aerosol insecticides come in metered dose delivery design, usually in the range of 3,000 sprays. Each spray is measured to deliver just the right amount of insecticide required to work effectively. The other good aspect of aerosol insecticides is that very small quantities are used as compared to other forms of insecticide application. This is due to the nearly gas (mist) form of the insecticide. The very low amounts involved ensure sub lethal dosage to humans thus being very safe for humans.

Challenges when using aerosols: One of the most common challenge in aerosol insecticide usage is that since the insecticide is delivered in a mixture of a propellant gas and insecticide mist ,these two forms easily get into respiratory system, eyes of people using them, in some instances causing adverse immunological responses reactions as well as, sneezing etc. Another challenge is that the insecticide's effectiveness depends on several conditions and one such condition is dosage of application. With aerosol it is very difficult to determine the right amount to be dispersed at a particular time as this amount depend very much on the volume of space in which the insecticide is to work and the susceptibility of the target organisms. All this has an effect on the efficacy of the insecticide. Metered aerosol cans to an

extent have helped to overcome the dosing quantity delivery determination challenge, and secondly making aerosols insecticide from a mixture of several types of insecticides including synergies is intended to overcome the susceptibility challenge.

2.1 Aerosol sprays composition

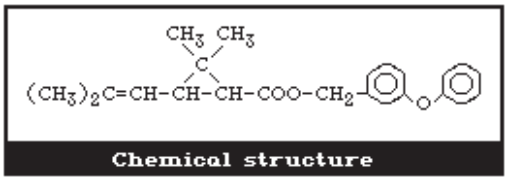
Even though the chemical composition of the aerosol sprays that are commercially sold is more or less the same, the amounts and types of pyrethroid isomers making up the aerosols differ from one brand to another hence affecting the efficacy. The formulated product contains many inert ingredients that can increase the toxicity of the product when compared to the technical-grade material. By law, the active ingredient must be identified by name on the pesticide label and its percentage must be provided. Non-active ingredients (sometimes called inert ingredients) do not need to be identified by name on a pesticide label, only the percentage of non-active ingredients must be specified, so it is often difficult to determine what other chemicals are included in the final formulated product (ATSDR. 2003).

All the aerosols tested in this study had the following isomers as part of their composition: imiprothrin, pralletrin and allethrin. The main difference among the aerosols was the presence of a pyrethroid that was not present in the others (Table 1). These included D-Phenothrin , a synthetic pyrethroid with high lethal activity against household insect pests, Tetramethrin, the second generation of synthetic pyrethroid, is a contact insecticide with a rapid knockdown action on flies (WHO, 2004). Manufacturer information showed that D-phenothrin is usually added at a higher rate (twice) that of tetramethrin which instead is combined with a synergist piperonyl butoxide. Piperonyl butoxide inhibits cytochrome P450 and esterase which are detoxification enzymes in insects. This inhibition paves way for elevated concentrations of active insecticide in the target animal for a longer period thus being lethal (Moores, et al. 2009).

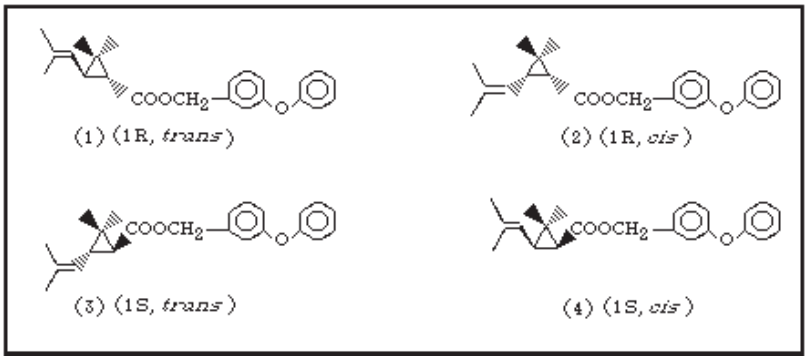
Aerosol constituent	Aerosol A	Aerosol B	Comments
Prallethrinin	0.4g/kg	0.34g/kg	
Imiprothrin	0.92g/kg	10g/kg	
D- phenothrin	0.92g/kg	-	
Tetramethrin	-	0.4g/kg	
Piperonyl butoxide	-	Present	Synergy : Cytochrome P450 and esterase inhibitor

Table 1. Aerosol Composition as identified from package label.

Details of particular components of interest:
Phenothrin
Molecular formula: $C_{23}H_{26}O_3$



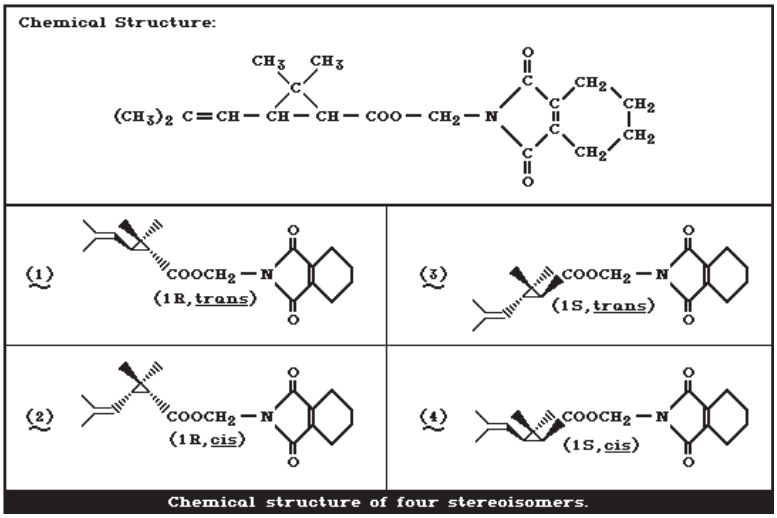
Racemic phenothrin was first synthesized by Itaya in 1969. It is prepared by esterifying (1RS, *cis,trans*)-2,2-dimethyl-3-(2,2-dimethylvinyl) cyclopropanecarboxylic acid (chrysanthemic acid) with 3-phenoxybenzyl alcohol,l (Fujimoto et al., 1973).
Phenothrin is thus a mixture of four stereoisomers. The cis:trans isomer ratio is 1:4 and the optical ratio of 1R:1S is 1:1 (racemic). the four isomers are present in the approximate ratio of 4:1:4:1 .. d-Phenothrin is a mixture of 2 isomers ,1R,cis,trans (i.e., . the cis:trans ratio being 1:4. The technical grade is 92.5-94.5% pure. The major impurities found in seven d-phenothrin preparations (average purity, 94.0%) are ethyl chrysanthemate (2.31%), 3-phenoxy-6-bromobenzyl *cis,trans*-chrysanthemate (0.66%), 3-phenoxytoluene (0.43%), and 4-phenoxybenzyl *cis,trans*-chrysanthemate (0.39%) (Miyamoto et al., 1984).



Chemical structure of the four stereoisomers of d-phenothrin.

Registry of Toxic Effects of Chemical Substances (RTECS) (1981-82 edition).

TetramethrinMolecular formula: $C_{19}H_{25}NO_4$
Chemical structure:

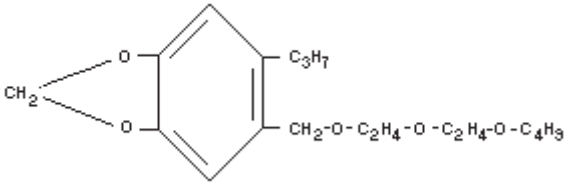


Chemical structure of four stereoisomers.

Tetramethrin was first synthesized in 1964 by Katoet. It is prepared by the esterificaton of (1RS,*cis,trans*)-2,2-dimethyl-3- (2,2-dimethylvinyl)-cyclo-propanecarboxylic acid (chrysanthemic acid) with 3,4,5,6-tetrahydrophthalimidomethyl alcohol.
It is a mixture of four stereoisomers The [1R,trans] isomer is the most active biologically of the isomers, followed by the [1R,cis] isomer. Registry of Toxic Effects of Chemical Substances (RTECS) (1981-82 edition).

Piperonyl butoxide

Chemical name: 3,4-methylenedioxy-6-propylbenxyl n-butyl diethyleneglycol ether
Synonyms
Alpha-[2-(2-n-butoxyethoxy)ethoxy]-4,5-methylendioxy-2-propyltolune or 6-(propylpiperonyl)-butyl carbityl ether or (3,4-methylenedioxy-6-propylbenzyl) (butyl diethylene glycolether) ether.
Empirical formula: $C_{19}H_{30}O_5$ (molecular weight 338)
Structural formula



Piperonyl butoxide is derived from piperic acid. Its synergistic activity is believed to be due to the presence of the methylenedioxy group in the molecular structure. Negherbon (1959). Piperonyl butoxide cannot be used as an insecticide alone. It is an effective synergist to pyrethrins and allethrin. The synergy effect is so pronounced that the resulting kill of insects is much greater than that which can be produced by pyrethrins alone. (FAO Meeting Report No. PL/1965/10/1 & WHO/Food Add./27.65)
This study looked at constituent isomeric influence on efficacy for two brands of commonly used aerosols in Southern Africa. Aerosol testing records for Southern Africa are hard to find, thus may indicate lack of aerosol insecticide testing in this region. Similar studies in other parts of the world have been carried out in simulated home environment, which to a larger extent affect the results as insecticide dispersal is affected by air circulation which is restricted by presence of furniture and other household items. Such studies are useful for assessing impact in home usage but not when focus is on the impact of the constituent isomers. To make it more important and acceptable this study used the peet glad chamber which ensures that there is no restricted air circulation, hence insecticide dispersal being uniform and unrestricted. The use of wild type mosquitoes collected from houses ensured that testing was conducted on vector population existing in the peoples' houses not the susceptible laboratory strain, hence more realistic situation.

2.2 Objectives

Even though the chemical composition of the aerosol sprays that are commercially sold is more or less the same, the amounts and types of pyrethroid isomers making up the aerosols differ from one brand to another, affecting the efficacy. The main objective of this study was

to compare knockdown as indicator of efficacy basing influenced by isomeric composition based on container label.

2.2.1 Methodology

Testing was done on space sprays (aerosol insecticides) that are readily available on the markets and mostly used in households. The testing followed World Health Organisation Pesticide Evaluation Scheme (WHOPES) standards as per requirement in aerosol testing. Testing was done in a Peet-grady chamber.

Culex pipiens mosquitoes were collected using an aspirator from random houses and were kept in a cage where they were fed with a sucrose solution for them to stay alive. The F1 generation from these mosquitoes was used in the study.

Adult mosquitoes were reared in constant temperature incubator. A plastic cup with a relatively moist filter paper was placed in the cage so that the mosquitoes had a favourable place to lay their eggs. Once the eggs were laid the filter paper was removed and placed in a container filled with distilled water so that they float off it. When they hatched the larvae was fed yeast. The larvae were kept in water troughs at 27^{o}C in an incubator. Pupae were placed in dishes so that adults emerged in the cages where they were placed.

Knockdown /Efficacy Tests

Standardized mosquito rearing and testing are essential to ensure the reliability and reproducibility of data. This is generally 27 ± 20C temperatures, relative humidity (RH) 80 ± 10% and photoperiod 12:12 hours (light: dark). WHOOPES's set procedures for aerosols testing in a Peet-grady chamber were followed. Space sprays were identified using letters not names. A common method used to determine the efficacy of a space spray uses the log probit analysis.

A total of 50 sugar fed 2-5 day old mosquitoes were placed in a small cage and placed in the chamber where there was a window for observation. Immediately before the test an automatic dispenser was shaken and aerosol was sprayed away from the chamber in a fume hood, for 3-5 seconds. Thereafter, 0.65±0.10g of the formulated product was sprayed, in a single application towards the centre, directly from the aerosol can. The number of mosquitoes knocked down was recorded every 10 minute intervals for a total of 60 minutes using a hand counter. The knocked down and all remaining mosquitoes were carefully transferred into separate clean holding cups. Mosquitoes were provided with 10% sugar solution on cotton wool and held for 24 hours at 27 ± 20C and 80 % ± 10% RH. The mortality was recorded 24 hours after exposure. The efficacy of a product is usually assessed using the minimum of three replicates and a control.

2.2.2 Results and discussion

Single blinding was used in this study in that the applicants did not know which brands of the aerosol they were administering. All cans were painted white. The space sprays that were used in this test were just identified as A and B. Aerosols A and B are the two most commonly sold on Malawian markets. A common method that is used to determine the efficacy of a space spray is known as a log probit analysis. This method describes the relationship between the time that has passed for the insecticide to induce knockdown in mosquitoes and the mortality at a prescribed dosage of the space sprays. It is a statistically derived average time interval during which 50% or 90% of a given population may be expected to die following acute administration of a chemical or physical agent (radiation) at a given concentration under a defined set of conditions(IUPAC, 1997).

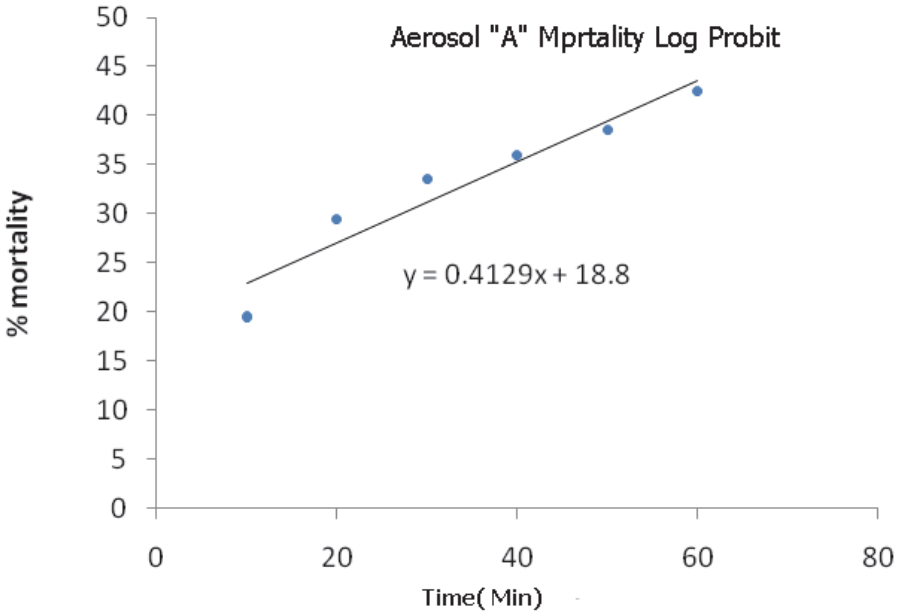


Fig. 1. Mortality Log probit after exposure to aerosol A.

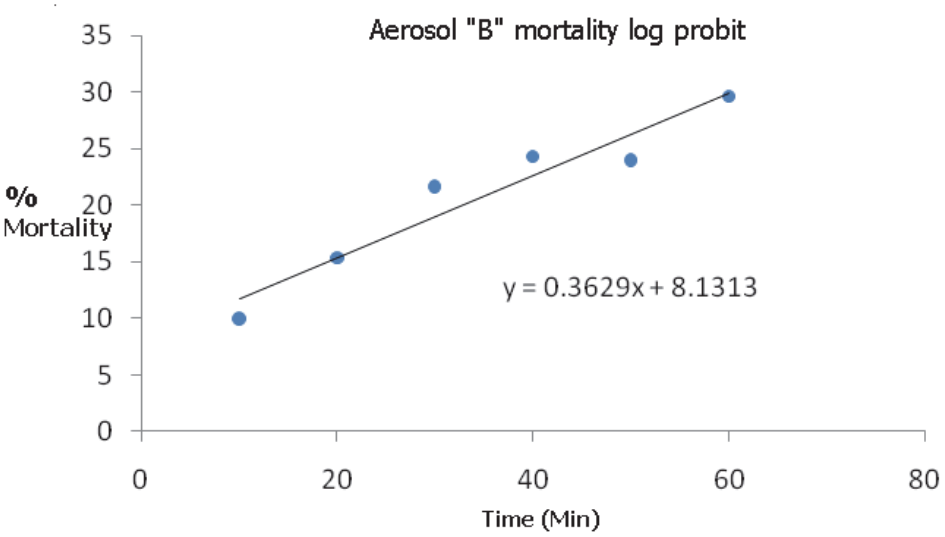


Fig. 2. Mortality Log probit after exposure to aerosol B.

Space spray	LT_{50} (P=0.05)	LT_{90} (P=0.05)
B	115.6 (109.956927-121.243073)*	226.15 (220.506927-231.793073)*
A	75.73(69.315463-82.144537)*	172.81(166.395463-179.224537)*

Table 2. Values are statistically significant LT_{50} and LT_{90}.

After 24 hrs the percent mortality for B was at 89% and that of A was 99%.
Using the log probit equations, the tabulation shows that LT_{50} for aerosol A is 75.73 minutes while that of aerosol B is 115.6 minutes. This means A and B takes these respective durations to kill half the population of the mosquitoes that were used per test. At 90% mortality of the mosquito population tested aerosol A again displays better efficacy in knocking down mosquitoes. The LT_{90} for A is 172.81 minutes while that for B is 226.15 minutes, t-test statistically significant ($P < 0.05$). The mosquitoes have shown a marked ability to withstand aerosol B unlike A. This could indicate a possible resistance or lower efficacy of the insecticide. In this case resistance would not be a possibility for the following reasons. The active ingridients in A and B have similar mode of action and belong to the same class of insecticide, thus if there was resistance to B as indicated by large LT values, cross resistance should also have been noted in A. The point of importance in this study is that there is not metabolic resistance as such the poor efficacy of B is not a result of metabolic resistance. The presence of a synergy piperonyl butoxide should have helped overcome any cytochrome p450 or esterase mediated metabolic resistance.
The isomeric composition of A includes the synthetic pyrethroids *Prallethrinin, Imiprothrin* and *D-phenothrin*. B is composed of the synthetic pyrethroids similar to those in A, *tetramethrin* and the synergist *piperonyl butoxide* however there is no D-phenothrin. . The two space sprays are different in that at least each has a pyrethroid that is not used in the other. *D-Phenothrin* is a synthetic pyrethroid with high lethal activity against household insect pests. *Tetramethrin*, is a second generation of synthetic pyrethroids, is a contact insecticide with a rapid knockdown action on flies (WHO, 2004). According to container labels *D-phenothrin* is added at 0.92g/kg whereas *tetramethrin* is added at 0.4g/kg per 300ml of the product. Prallethrin in A is added at 0.4g/kg while in B it is added at 0.34g/kg. Imiprothrin is at 0.92g/kg in A and in B it is at 10g/kg.
The chemical compositions show that *D-phenothrin* is An importantpyrethroid isomer since it is the component that differentiates A from B. It is added at a higher concentration than its corresponding isomer, *tetramethrin* and as previously stated it is very lethal to mosquitoes. All these factors must contribute to the lower LT values for A . Another important point is thatall constituent pyrethroids except imiprothrin are added at a higher concentration in A than in B , supporting the higher efficacy that has been exhibited by A. While it has been concluded that the use of *piperonyl butoxide* and *tetramethrin* with synthetic pyrethroid insecticides provides the best results for the control of house flies, it does not seem to be quite an effective combination in the case of mosquitoes as Cakir indicated (Cakir et al, 2008) as also indicated in this study.
Another factor of interest is the addition of imiprothrin at a very higher rate than other components 10g/kg in B as compared to A's 0.92g/kg. The first one being that imiprothrin

is not important isomer and can be left out. The second possibility is that the manufacturer of aerosol B realizes that the constituents used were not that efficacy and decided to provide a very higher proportion of this particular component to make up for the low efficacy which would be in effective at low dosage. The addition of a synergy to slow down insecticide metabolism support the idea that components of Product B are of less efficacy or the dosage of the potent component provided is *under* dose as such needed to be enhanced(boosted) by other components.

Aerosol A has three active ingredients, while B has four active ingredients. Yet B has a lower efficacy rate as compared to A. Having more pyrethroid constituents or active ingredients should not be taken as a sign of a better insecticide. It could as in this case act as pointer of weakness. Another point of interest could be that a product label indicating more components could be indicative of weakness in the components and more are added to shore up the product performance.

The presence of a synergy where resistance is not reported should also act as a red flag on the efficacy of the insecticide's active components efficacy.

3. Conclusion

The study has confirmed what WHO indicates on pesticide database that pyrethroid isomer D-phenothrin is very potent when applied to dipteral members. Fast acting knockdown pyrethroid like tetramethrin need to be encouraged but the dosage should be at optimum recommended not lower dosages as in product B as this may easily result in the built up of resistance. The fact that a synergy is in cooperated when such low dosages are used should not be used as a reason that resistance my not arise. As the study has shown the product still remain inefficacy. Country regulatory bodies should set a minimum concentration at which every isomer/component should be added in aerosol insecticides, below which such products should not be.

4. Acknowledgment

Our gratitude to Twalibu Tandwe, Raphael Kondwani and Martin Chiumia for their support in mosquito collection, raring and data collection in the laboratory. Special mention goes to The Entomology section of the Department of Biological Sciences of Chancellor College of the University of Malawi and for allowing use ofthe insectary and equipment.

5. References

Agency for Toxic Substances and Disease Registry (ATSDR).. *Toxicological profile forpyrethrins and pyrethroids*. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service. (2003) http://www.atsdr.cdc.gov/ToxProfiles/tp155-c1-b.pdf

Bonnet, J., Pennetier, C., Duchon, S., Lapied, B., and Corbel, V., *Multi-function oxidases are responsible for the synergistic interactions occurring between repellents and insecticides in Mosquitoes,* Parasites and Vectors (2009), 2-17

Cakir, G., O. Yavuz, O., and Kocak, O., *Effects of Piperonyl Butoxide and Tetramethrin Combinations on Biological Activities of Selected Synthetic Pyrethroid Insecticides against Different Housefly (Musca domestica L., Diptera: Muscidae) Populations,* Acta Vet. Brno, 2008, 77: 467–474

Diabate et al, *The role of agricultural insecticides in resistance to pyrethroids in Anopheles Gambiae S.L in Burkina Faso Am. J. Trop. Med. Hyg.,* 67(6), 2002, pp. 617–622

Fradin, M. S., Mosquitoes *and Mosquito Repellents: A Clinician's Guide,* 128(11), (1998) 931-940, Annals of Internal Medicine

Horton, M.K., Rundle, A., Camann, D.E., Barr, D.B., Rauh, V.A., Whyatt, R.M., *Impact of Prenatal Exposure to Piperonyl Butoxide and Permethrin on 36-Month Neurodevelopment. Pediatrics*, 2011; DOI: 10.1542/peds.2010-0133IUPAC *Compendium of Chemical Terminology* 2nd Edition (1997)

Khadri MS, Kwok KL, Noor MI, Lee HL (2009) *Efficacy of commercial household insecticide aerosol sprays against Aedes aegypti (Linn.) under simulated field conditions.* Southeast Asian J Trop Med Public Health. ;40(6):1226-34.

Marcombe, S., Carron A, Darriet, F., Etienne, M., Agnew, P., Tolosa, M., Yp-Tcha, M.M., Lagneau, C., Yébakima, A., Corbel, V., *Reduced efficacy of pyrethroid space sprays for dengue control in an area of Martinique with pyrethroid resistance.* Am J Trop Med Hyg. 2009 May;80(5):745-51

Moores, G. D., Philippou, D., Borzatta, V., Trincia, P., Jewess, P., Gunning, R., Bingham, G. (2009). "*An analogue of piperonyl butoxide facilitates the characterisation of metabolic resistance*". Pest Manag. Sci. 65 (2): 150–154. doi:10.1002/ps.1661. PMID 18951 417

Nazni, W.A., Lee, H.L. and Azahari, A.H., *Adult and larval insecticide susceptibility status of Culex quinquefasciatus (Say) mosquitoes in Kuala Lumpur Malaysia,* Tropical Biomedicine 22(1), 2005.,pp 63–68

Olga Eremina. *Investigation Of Several Aerosol Insecticide Compositions Containing Synergists.* Proceedings of the 4th international conference on urban pests (2002)Scientific Research Institute for Disinfectology, 18, Nauchny proyezd, Moscow, 117246, Russia

Pemba, D.F, (2008), *Mosquito species and sensitivity to insecticides in Liwonde town and Liwonde National park,* unpublished

Pennetier, C., Corbel, V., and Hougard , J.R., *Combination of a non-pyrethroid Insecticide and a repellent: A new approach for controlling knockdown resistance mosquitoes,* Am. J. Trop. Med. Hyg., 72(6), 2005, pp. 739-744, The American Society of Tropical Medicine and Hygiene

Pridgeon, J.W., Bernier, U.R., and Becnel, J.J., *Toxicity Comparison of Eight Repellents against four female mosquito species,* Journal of the American Mosquito Control Association, 25(2):168–173, 2009, The American Mosquito Control Association, Inc

Rapeeporn Y, Rachada K, Chareonviriyaphap T, and Rongnoparut P, *Characterization of deltamethrin resistance in field populations of Aedes aegypti in Thailand,* Journal of Vector Ecology 30 (1),2005, pp 144-150

WHO, (2000), *malarial control:* Insecticides *for indoor residual spraying,* WHO pesticides evaluation schemes (WHOPES)

WHO, (2009), *Guidelines for efficacy testing household insecticide products,* WHO pesticides evaluation schemes (WHOPES)

WHO, (1996), *Report of the WHO informal consultation of the evaluation and testing of insecticides*, WHO pesticides evaluation schemes (WHOPES)

Xue, R., Ali, A., and Barnard, D.R., *Laboratory Evaluation of toxicity of 16 insect repellents in aerosol sprays in adult mosquitoes,* Journal of The mosquito control association,19(3),(2003),271-274

27

Bacillus sphaericus and *Bacillus thuringiensis* to Insect Control: Process Development of Small Scale Production to Pilot-Plant-Fermenters

Christine Lamenha Luna-Finkler and Leandro Finkler
Federal University of Pernambuco
Brasil

1. Introduction

Bacteria from the genus *Bacillus* are among the entomopathogenic microorganisms most commonly used as biocontrol agents. Among them, *Bacillus sphaericus* (Bs) and *Bacillus thuringiensis* (Bt) are used in vector control programs of endemic diseases such as dengue, malaria and filariasis. Because they are spore-former bacteria and produce toxins highly specific to target insects are better suited to industrial production and field application.
Development of microbial insecticides production technology depends basically of four steps: i) isolation and selection of strains with higher activity, ii) fermentation process optimization using cheap raw materials, iii) application of an appropriate method to cell separation; iv) development of formulations.
The aims of this chapter are present a general overview of properties and applied use of these mosquitocidal bacteria and strategies or considerations to development a cost-effective process for success of its large scale production.
The chapter will be divided into 3 (three) stages:

- - *Bacillus sphaericus* and *Bacillus thuringiensis* in vector control: an overview
- - Stages of product development: storage and maintenance organisms, fermentation methods, solid-liquid separation techniques and formulations.
- - Process on a laboratory scale to pilot-plant-fermenters.

2. *Bacillus sphaericus* and *Bacillus thuringiensis* in vector control: an overview

The indiscriminate use of chemical pesticides and their harmful consequences to the environment encouraged the development of biological control techniques against harmful insects, both in agriculture and public health. Among the procedures used for integrated pest management, biological control techniques are frequently chosen by the advantages they offer, particularly specificity and safety for man.
Among the biological control agents, bacteria of the genus *Bacillus* are among the most widely used entomopathogenic microorganisms, especially for its ability to form spores and toxins highly specific to target insects. Bs and Bt are employed in programs to control larvae of important mosquito disease vectors such as filariasis, malaria and dengue.

Bs was first discovered in 1904, but only in 1965 its activity against larvae of Culicidae was recognized. However, the strain discovered (*B. sphaericus* K) did not have a very effective activity, which limited its use (Kellen et al., 1965). Currently, several toxic strains are known, and most studies utilize strains 1593 and 2362, respectively isolated in Indonesia (Singer, 1973) and Nigeria (Weiser, 1984). It has high specificity and toxicity against insects of the Order Diptera, especially of genus *Culex* and *Anopheles*. Because it is harmless to humans, animals and the environment, its use is recommended by the World Health Organization in public health programs.

Bs is a Gram-positive bacterium, strictly aerobic and is not able to use sugars as carbon sources and energy, requiring growth media containing proteins and ions Ca^{++} and Mg^{++} for sporulation (Russel et al., 1989). Present spherical, terminal or sub-terminal spores and swollen sporangia, and the most toxic strains produce a protein crystal in the form of parallelepiped composed of two proteins of 51 and 42 kDa. Both proteins are required for the toxic action, being synthesized in equimolar amounts during sporulation (Charles et al., 1997). After sporulation, the crystal remains associated to endospore, and this complex (endospore + crystal) is inside the exosporium.

After ingestion of the spore/crystal complex, proteins are solubilized in the larvae stomach by the combined action of proteases and alkaline pH, causing damage to the nervous system and digestive tract, until the occurrence of sepsis. The period between ingestion of the toxin and lethality of larvae is up to 48 hours.

Bs is widely distributed throughout the environment and can be isolated from soil, aquatic ecosystems and in dead mosquito larvae. It has the ability to persist in aquatic environments, polluted or not (Baumann et al., 1991). This represents a great advantage when it is applied in environments containing large amounts of organic matter, providing a more durable control on larvae populations.

Bt was first isolated in Japan, when Ishawata described a spore-forming bacteria that caused mortality in larvae of the silkworm (*Bombix mori*). In 1911, Berliner reported the same type of bacteria acting on the flour moth (*Anagasta kuhniella*), and in 1915 named it *Bacillus thuringiensis*. The researcher said the presence of an inclusion body in spore, but not related to the insecticidal properties of the microorganism, mentioning the possibility of using it to control moths. Their use to control Lepidoptera was soon recognized, and in 1938 was marketed the first product based on this bacterium (Sporeine), effective against caterpillars of various vegetables and fruit (Dias, 1992).

Products development based on Bt has intensified in the 50's, but only in 1970 the strain *B. thuringiensis* var. *kurstaki* HD-1 has been produced commercially by many companies and agrochemical products of fermentation (Navon, 2000). Currently, these bacteria-based products account for about 90% of the worldwide market for biological control agents.

In 1977, Goldberg and Margalit (1977) identified a strain that showed toxic activity against Diptera. The strain was isolated in Israel from moribund larvae of *Culex pipiens*, being called *B. thuringiensis* var. *israelensis* (Bti), which is the first bacterium used in biological control programs against Diptera around the world. In Pasteur Institute this strain was identified as *B. thuringiensis* serotype H-14. It had high toxicity to larvae of mosquitoes of genus *Aedes* and *Culex*, and less active against *Anopheles*. It can be easily found in the environment and isolated from soil, warehouses, surface of leaves and insect habitat (Hongyu et al., 2000). It has aerobic metabolism, glucose is used as a source of carbon and energy, as well sucrose, L-arabinose, D-xylose and D-mannitol.

The safety of products based on Bt on non-target organisms has been extensively reviewed by several authors. It is completely harmless to humans and other mammals, as well as aquatic vertebrates, invertebrates and plants.

Insecticidal activity of most Bt subspecies is related to the production of a parasporal inclusion of the crystal structure called δ-endotoxin, which is synthesized during sporulation and is located associated with the spore. The crystal structure is comprised of three distinct domains: Domain I (a 7 α-helical bundle) is equipped for pore formation in insect epithelial membrane; Domain II (a triple β-sheet structure) may be responsible for receptor recognition; Domain III (a β-sandwich region) may protect the toxin from further degradation during proteolytic processing (Honée and Visser, 1993; Vontersch et al., 1994).

Depending on the variety of species, the δ-endotoxins are composed of different structures and molecular weights ranging between 27 and 160 kDa. These proteins (protoxins) are called Cry proteins and are identified according to their degree of toxicity to the various orders of insects susceptible. Table 1 shows examples of Cry proteins found in Bt, which can be divided on the basis of their activity into five major classes: i) Lepidopteran specific; ii) Lepidopteran and Coleopteran specific; iii) Coleopteran specific; iv) Dipteran specific; and v) Nematode specific (Cannon, 1996).

When larvae ingest these inclusions, protoxins are solubilized and converted into active toxins of low molecular weight by enzymes (proteases) in the larvae stomach at alkaline pH. After binding to specific receptors, toxin rapidly enters in the plasma membrane of intestine cells, with formation of pores channels and the loss of membrane integrity. Such events lead to cell lysis and finally the death of the insect through starvation or septicemia (Kumar et al., 1996).

Protein	Subclass	Protoxin (kDa)	Target insect
CryI	IA(a)	130-160	Lepidopteran
	IA(b)	130-160	Lepidopteran/Dipteran
	IA(c)	130-160	Lepidopteran
	IB	130-160	Lepidopteran
	IC	130-160	Lepidopteran
	ID	130-160	Lepidopteran
	IE	130-160	Lepidopteran
	IF	130-160	Lepidopteran
CryII	IIA	70-71	Lepidopteran/Dipteran
	IIB	70-71	Lepidopteran
	IIC	70-71	Lepidopteran
CryIII	IIIA	73	Coleopteran
	IIIB	73	Coleopteran
	IIIC	73	Coleopteran
	IIID	73	Coleopteran
CryIV	IVA	134	Dipteran
	IVB	128	Mosquitoes
	IVC	58	Blackflies
	IVD	72	Nematodes
	CytA	27	
CryV	V	81.2	Lepitopteran/Coleopteran

Table 1. Cry proteins found in *B. thuringiensis*, molecular sizes and target insect (adapted from Rukmini et al., 2000).

3. Stages of product development

3.1 Storage and maintenance organisms

Bacillus cultures should be kept in conditions which ensure their phenotypic and genotypic characteristics. In an industrial fermentation of *Bacillus* species, it is important to ensure the integrity of the strain, and the use of appropriate methods of culture preservation is fundamental to the development of bioprocess.

Not all species respond similarly to preservation methods, and its success depends on appropriate choice of medium, the culture procedures and storage time. Is recommended verify periodically the intactness of bacterial plasmid content in all steps of culture, because changes in the plasmid content could lead the production of non-active culture in industrial fermentation.

Some of the main methods for the preservation of *Bacillus* strains are strips of filter paper, periodic subcultures, mineral oil and freeze-drying.

3.2 Fermentation methods

Cultivation of entomopathogenic bacteria are carried out in batch or fed batch. In a typical batch fermentation process, cultures of Bs and Bt are characterized by the following morphological and physiological variations: a) Vegetative growth stage, with the occurrence of exponential phase, presence of isolated cells, in pairs and in chains, with uniform size and high mobility; b) Transition to sporulation, with decreased growth rate and presence of shorter and isolated cells without motility; c) Stage of sporulation; d) Stage of spore maturation and cell lysis. In Bt cultures is observed a linear growth phase after exponential growth phase, with rapid drop in pH. In the stage of sporulation, there is a tendency to cells flocculation.

Proper choice of the medium ingredients is critical to success of commercial production, aiming to obtain a greater toxic activity per volume of fermentation broth. The medium selection depends on three factors: assimilation by the organism, availability and cost. To obtain large-scale bio-insecticides, the use of raw materials from industrial waste can reduce the costs of the fermentation process, but care should be taken into account the influence of media components on cell recovery and formulation of the final product.

The main components of culture media are carbon sources, nitrogen and trace elements. A large number of agro-products and waste could be used as substrates, for example, molasses, water washing and pressing of fruit and cereals, cheese whey, peptones and slaughterhouse waste, oil cakes, fish meal, among others (Dias, 1992). In a previous study (Luna et al., 2004), was evaluated use of supernatants obtained from the flocculation/sedimentation process to cell separation of *B. thuringiensis* var. *israelensis* – Bti, which were supplemented with original culture medium. Spores concentration of $1x10^{10}$ UFC/mL were obtained, demonstrating the viability to use the supernatant to formulation of the culture medium.

Bs does not use carbohydrates as carbon source, and media should be formulated based on protein components. The choice of substrates for Bti cultivation is simpler because it consumes sugars, amino acids and proteins. Glucose, starch and molasses are the carbon sources most commonly used. Medium for the cultivation of this bacterium must be formulated properly on the concentration of sugar, because the use of high levels of this substrate without the appropriate adjustment in the concentration of nitrogen source could cause a drop in pH values between 5.6 and 5 8, which could lead to inhibition of sporulation.

The main parameters monitored during the fermentation process are temperature, dissolved oxygen, pH and sugar concentration. Due to the large consumption of oxygen during the cultivation, control of this parameter is very important and should not reach values below 20% (Couch, 2000).
Controversy exists regarding the need for pH control during fermentation, and in some cases are used buffered media for not having an adequate system of control. Nevertheless, studies had been identified an increase in the insecticidal activity of Bs (Yousten and Wallis, 1987) and Bti (Smith, 1982) when fermentation was performed without pH control.
The typical pattern for the change in pH in Bti cultivation reflects acid production in the first hours of culture from the use of glucose or others sugars. Then, pH gradually increases by the production of nitrogen compounds, reaching values close to 8.0 after about 30 hours and 9.0 after 50-60 hours of cultivation. In contrast, pH in the Bs fermentation increases gradually along the growth and spore formation, since there is no acid formation by the absence of sugars as a carbon source. In this case, an accumulation of ammonia is probably due to deamination of amino acids, and pH values are between 8.0 and 9.0, depending on the protein content in the culture medium.
In fed batch cultivation of Bti, the amount of sugar should not reach levels less than 2 g/L. Temperature should be maintained at 30°C ± 2°C; higher values inhibit toxins production while values below 25°C increase production costs.
Higher spores concentrations in Bt and Bs cultivations are required to obtain a high larvicidal activity, because sporulation is associated with the crystal toxic synthesis. However, high levels of sporulation are not always associated with a high toxicity of the crystals produced.

3.3 Solid-liquid separation techniques

About the product recovery step, the main interest in the production of biological insecticides is the cell mass and content of toxins. Thus, the main focus is solid-liquid separation. Removal of microbial cells is one of the most challenging problems in solid–liquid separation. These particles having dimensions of the order of microns and low density, and often require pre-treatment to obtain efficient recoveries in the separation process by filtration or gravitational sedimentation, such as flocculation process.
In studies investigating the use of coagulants agents for cells recovery of Bs and Bti, it was observed that addition of electrolytes such as $CaCl_2.2H_2O$, $FeCl_3.6H_2O$ and $Al_2(SO_4)_3$ was efficient for cells recovery in concentrations ranging from 0.1 to 0.35 g/L for recovery efficiencies above 95%. Flocculation at pH between 2 and 4 promotes cellular aggregation for both bacteria. Characterization of flocculent suspensions demonstrated average diameter of flocs is dependent of the flocculated suspension (medium, pH) and the mechanism of flocculating agent adsorption (electrolytes or pH changes) to cells. Overall, diameters were ranging from 103.1 ± 4.3 μm and 275.6 ± 9.5 μm to Bs and 162.2 ± 21.4μm and 313.9 ± 21.9 μm to Bti. Major values were corresponding to media containing suspended solids (Luna-Finkler & Finkler, 2008; Luna et al., 2003).
Addition of inorganic electrolytes can alter the charge properties of suspensions, making them unstable and causing the agglomeration of particles. Some factors influence the stability of colloidal dispersions, and therefore can determine the flocculation, such as electrolyte concentration (critical coagulation concentration), nature of the suspension, hydrolysis species in solution and ion valence of opposite charge to particles. Ions can act as

electrolytes, reducing the electrostatic repulsion that usually exists between colloidal particles, or form bridges between them.
There are few studies about pH influence on the flocculation of bacteria. Luna et al. (2001), investigating the flocculation of Bs, found that pH 3.0 favored the aggregation and cells hydrophobicity. In general, most bacteria have a net negative charge on their surfaces, presenting isoelectric point in an acid medium. This is probably due to the large percentage of anionic groups, especially carboxyl and phosphate, to the detriment of cationic. Thus, the negatively charged surface allows interaction between cells and the protons in the environment, enabling the aggregation.
The observation that bacterial cells have a natural tendency to adhere to air bubbles during submerged and aerated fermentation processes suggests using the flotation for biomass recovery. The natural formation of foam is very common in these processes, due to the presence of protein in the original composition of the culture media or originated from the metabolism or cell lysis, either by production of surfactant substances during fermentation. This separation procedure is a technique well established in mineral technology and can be used for the recovery of various types of biological materials. The use of this process in the spores recovery of *Bacillus* genus is a promising alternative for obtaining bioactive concentrate, especially considering the hydrophobic nature of these cells.
Tests using a mechanical flotation cell showed spores recovery of Bs close to 100% for a flotation time of 5 minutes in the presence of a cationic collector and agitation of 960 rpm. Column flotation tests were performed in batch without addition of reagents, with a residual spores concentration of 2.5% after 30 minutes (Luna et al., 2005).

3.4 Formulation

Cells concentrated of the bacterial insecticide of genus *Bacillus*, after separated from their fermentation broths, are composed of a complex mixture of protoxins and spores. As proteins, protoxins are more sensitive to changes in their chemical structures and could be inactived by microbial contamination, ultraviolet radiation, proteolytic enzymes, sensitivity to temperature conditions, toxic compounds, uncontrolled drying or moisture.
These problems can be overcome by the development of appropriate techniques for product formulation. Formulated products increase efficiency in the field, are made for easy handling and application and allow lower-cost storage, reducing the loss in quality. However, there are few reports in the scientific literature on the study of formulations of entomopathogenic microorganisms, especially considering that formulations are generally kept confidential by the companies.
The components of a formulation should be inactive to the larvae and involve protoxins without increasing particle size (no more than 12 μm), otherwise could not be digested by the insect. Moreover, should contribute to increasing stability, virulence, efficacy and persistence of the biocontrol agent. Most pathogens of insects are highly susceptible to sunlight and require further protection methods to prolong its activity. It is essential to the improvement formulations based on entomopathogenic bacteria, especially research that characterizes relations between the components of a formulated (pathogen-adjuvant) and the factors that interfere with their production (temperature, humidity, etc.).
Formulations must also be compatible with the application techniques and existing equipments. In general, the application of microbial insecticides has been carried out with equipment and technology developed for chemical insecticides. Otherwise, would be difficult to compete in the market. Products based on entomopathogenic *Bacillus* can be

liquid formulations, solids (wettable powders, dusts, water dispersible granules, encapsulated forms) and oil emulsions. A solid formulation has, in general, greater stability when compared to a liquid formulation, due to low moisture content, and also favors transportation and storage.
The first step in developing of a solid formulation is the powder preparation, which comprises the steps of drying and pulverizing the active ingredient. Determination of parameters as residual moisture, particle size distribution, angle of repose, flow time, volume and apparent and compacted density are indispensable for characterize the active powder.
For the encapsulated formulations, the choice of the encapsulating agent depends on the method of encapsulation, the type of product application and its mechanism of action. The rate of polymers hydrolysis depends on their chemical composition, the proportion of monomers and the particle size, and encapsulated cells may be released by mechanical stimuli (breaking pressure) or others, such as changes in temperature or pH.
Methods employed for microencapsulation can be physical-chemical (evaporation/solvent extraction, phase separation or coacervation, liposome involvement), physical (spray drying, freeze drying, fluidized bed) and chemical (molecular inclusion, interfacial polymerization). The selection of preparation method depends on the properties of the polymer and the active ingredient.
The use of microcapsules for the development of microbial insecticides formulations has some advantages: protecting from adverse environmental conditions, increases shelf life of the product, controls the release, modifies undesirable properties of the active principle, form solid systems and releases the active ingredient in the desired location.

4. Process on a laboratory scale to pilot-plant-fermenters

In bioprocess development there are different operating conditions of bioreactors. Batch fermentation is currently used to *Bacillus thuringiensis* cultivation (Rivera et al., 1999; Silva et al., 2011; Vu et al., 2010). However, there are studies about cultivation in continuous (Selinger et al., 1988; Mignone & Avignone-Rossa, 1996) and fed-batch (Jong et al., 1994; Jong et al., 1995; Jing-Wen et al., 2007) conditions. Also, cultivation in solid-state fermentation (Capalbo et al., 2001; Vimala-Devi et al., 2005) and pneumatic agitation-aeration (air-lift) (Huang et al., 2001) has been recently investigated.
In all these processes control variables must be well established, mainly when the purpose is up-scaling fermentation processes from lab-scale to commercial units. Culture medium volume is increased and phenomena of mass transfer and energy become more complex. The main variables that influence on cell growth and metabolites production are pH, temperature, dissolved oxygen (DO), composition of culture medium, oxygen transfer rate (OTR) and heat transfer rate (Hsu & Wu, 2002). At large scale cultivation of *Bacillus* species it is critical to ensure that oxygen transfer and cooling capacity be adequate (Yang & Wang, 1998). Other factors of relevance for scale up are quality of mixing, shear stress, selection of cheaper media, foam control, physiological state of the inoculum and sterilization of culture medium (Humphrey, 1998).
In scale-up of fermentation processes is essential to determine criteria and factors that reflect critical parameters on the process. For *Bacillus* fermentation, which is an aerobic bacterium, the most utilized criteria for scale-up is the correlation between the volumetric oxygen transfer coefficient (K_{La}) and the volumetric airflow rate per unit volume (Q/V). Geometric

similarities are another important factor, which represents the ratio between liquid height and vessel diameter or the ratio between impeller diameter and vessel diameter. This parameter is important from the practical point of view, because it simplifies prediction of large-scale fermentor performance (Ju & Chase, 1992).

These concepts were adopted for some researchers in their studies. As the *Bacillus* cultivation occurs in aerobic conditions at pH and temperature defined, different strategies were established to scaling up. A general alternative observed in different studies is the use of renewable resources and readily available as raw materials for culture media composition. Moreover, also is possible make use of cultivation strategies to reduce the bioreaction time as inoculum volume, aeration rate and bioreactor design. Some examples of strategies adopted to increase yields in cellular mass and endotoxin production are presented at Table 2.

Objective	Strategy	Microrganism	Results	Reference
Formulation of fermentation media for production of δ-endotoxins from *Bacillus thuringiensis*	Leguminous seeds as horse beans, kidney beans, lima beans, soybeans, chick peas, lentils, and peanuts were incorporated in fermentation media as sole proteins sources for biosynthesis of the endotoxins. The cotton pests, *Spodoptera littoralis, Spodoptera exigua*, and *Heliothis armigera* were used as test insects for biological assays	*Bacillus thuringiensis* subsp. *kurstaki* and *entomocidus*	*B. thuringienis* subsp. *kurstaki* produced from media containing these nutrients killed 80–100% of *H. armigera* larvae when tested at 500 µg/ml diet.	Salama *et al.* (1983)
Effect of media formulation and culture conditions on growth, sporulation and endotoxin production	Use of process involving two inoculum stages and a 48h production stage in a 40 L fermenter	*Bacillus thuringiensis* subsp. *israelensis*	The viable cell count of 6.5 x 10^9/ml with greater than 95% sporulation	Pearson & Ward (1988)

Table 2. Studies about environmental conditions that have effect on the *Bacillus* cultivation processes.

Objective	**Strategy**	**Microrganism**	**Results**	**Reference**
Growth, sporulation and δ-endotoxin production in oxygen limited and non-limited cultures	Production of crystals and spores was studied under different aeration conditions	*Bacillus thuringiensis* var *israelensis*	δ-endotoxin yields diminished under O_2 limitation, suggesting that toxin synthesis mechanism could have been affected.	Avignone-Rossa et al. (1992)
Cultivation in an airlift reactor with wire mesh draft tubes	An aeration strategy was proposed for foam control in an airlift reactor with double wire mesh draft tubes	*Bacillus thuringiensis*	The production of thuringiensin based on the proposed strategy was up to 70% higher than that of using the conventional cultivation method with addition of antifoam agents for foam control	Huang *et al.* (2001)
Application of a simple yeast extract from spent brewer's yeast for growth and sporulation	The suitability of using a simple brewer's yeast extract (BYE), prepared by autolysis of complete beer slurry, was studied in baffled shaken flasks	*Bacillus thuringiensis* subsp. *kurstaki*	Growth in the medium with a commercial laboratory-grade yeast extract was better than autolysed.	Saksinchai et al. (2001)
Effect of shear stress for thuringiensin production	Agitation speed and aeration rate were varied during the exponential growth phase and stationary phase	*Bacillus thuringiensis*	By decreasing the agitation speed during the stationary phase, product formation was increased up to 43%.	Wu *et al.* (2002)
Barley-based medium for the cost-effective production	Using barley *Hordeum vulgare* as the carbon source led to the development of a protocol for the cost-effective, mass production of Bt.	*Bacillus thuringiensis*	Feeding cessation of the larvae followed by 85% and 100% mortality by 48 and 72 h after treatment	Vimala Devi *et al.* (2005)

Objective	Strategy	Microrganism	Results	Reference
Production of mosquitocidal *Bacillus sphaericus* by solid state fermentation using agricultural wastes	Twelve agricultural wastes were tested as the main carbon, nitrogen and energy sources under solid state fermentation	*Bacillus sphaericus* NRC 69	Wheat bran was the most efficient substrate for the production of *B. sphaericus* mosquitocidal toxins against larvae of *Culex pipiens* (LC_{50} 1.2 ppm)	El-Bendary (2010)
Development of a cost-effective medium for the large scale production	Use soybean flour (*Glycine max*), groundnut cake powder (*Arachis hypogea*), and wheat bran extract (*Triticum aestivum*)	*Bacillus thuringiensis* var *israelensis* (*B.t.i.*)	Maximum toxicity (LC_{50} 8.89 ng/ml against *Culex quinquefasciatus* IIIrd instar larvae), highest spore count (0.48×10^{11}c.f.u./ml), and maximum biomass (7.8 g/L) after 24 h.	Prabakaran & Balaraman (2006)
Scale-up based on oxygen transfer	Fermentation process scale-up on the basis of the product K_La x p, where p is the fermentor total pressure	*Bacillus thuringiensis*	Fermentation time was decreased while biomass yield and sporulation efficiency were unchanged	Flores *et al.* (1997)
Stirred tank for δ-endotoxin production: mathematical modelling and scaling-up studies	Soya and molasses were used in the medium culture. Aeration, agitation, pH and molasses initial concentration were chosen as experimental factors	*B. thuringiensis* H-14	The mathematical model revealed that the optimal batch cultivation conditions with respect to agitation, pH, and initial concentration of molasses were 325 rpm, 7.1 and 2.1% (w/v), respectively.	Abdel-Hameed (2001)
Scale-up of biopesticide production processes using wastewater sludge as a raw material	Use of shaken flasks and two geometrically similar fermentors (15 and 150 L)	*B. thuringiensis* var. *kurstaki* HD-1 (ATCC 33679)	High productivity for toxin protein yield and high protease activity.	Yezza *et al.* (2004)

Table 3. Studies about scale-up strategies to *Bacillus* cultivation.

Concerning the large scale production, some researchers have developed studies about strategies to increase of the bioreactor size. For this, some variables should be considered. In the Table 3 are presented assays that use environmental conditions to proceed the bioprocess scale-up.

5. Conclusion

Bacteria of the genus *Bacillus* are among the most widely used entomopathogenic microorganisms, especially for its ability to form spores and toxins highly specific to target insects. Because they are spore-forming bacteria, are better suited to industrial production and field application, because they have less sensitivity to ultraviolet radiation and adverse weather conditions, increasing their persistence in the field.

Bacillus sphaericus and *Bacillus thuringiensis* are important agents utilized in insect control programs to reduce the population of disease vector species that transmit diseases such as malaria, yellow fever and dengue. Its cultivation of small scale production to pilot-plant-fermenters requires knowledge about the stages of product development: storage and maintenance organisms, fermentation methods, solid-liquid separation techniques and formulations.

The process expansion on a laboratory scale to pilot-plant fermenters requires perform lab-scale experiments to support process changes and that the cost-benefit analysis is feasible. To achieve this purpose, studies have been developed using different strategies to defining the bioreactor design, the raw material used to compose the culture medium and cultivation conditions. Implementing scaling-up procedures is always a challenge; however, many nations are requiring the definition of working conditions for the cultivation of *Bacillus* species in large scale aiming application these organisms to remedy endemic diseases that affect the public health. For that, it is necessary the development of new technologies to expanding the commercial production to increase the availability of these products in the world market.

6. Acknowledgment

The authors are grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) and Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES) for the financial support. This work is dedicated to memory of professor Giulio Massarani.

7. References

Abdel-Hameed, A. (2001) Stirred Tank Culture of *Bacillus thuringiensis* H-14 for Production of the Mosquitocidal δ-endotoxin: Mathematical Modelling and Scaling-up Studies, *World Journal of Microbiology and Biotechnology,* Vol.17, No.9, (December 2001), pp. 857-861, ISSN: 0959-3993

Avignone-Rossa, C.; Arcas, J. & Mignone, C. (1992) *Bacillus thuringiensis* Growth, Sporulation and δ-endotoxin Production in Oxygen Limited and Non-limited Cultures, *World Journal of Microbiology and Biotechnology*, Vol.8, No.3, (May 1992), pp. 301-304, ISSN 0959-3993

Baumann, P.; Clark, M.A.; Baumann, L. & Broadwell, A.H. (1991). *Bacillus sphaericus* as a Mosquito Pathogen: Properties of the Organism and Its Toxins. *Microbiology and Molecular Biology Reviews*, Vol.55, No.3, (September 1991), pp. 425-436, ISSN 1092-2172

Cannon, R.J.C. (1996) *Bacillus thuringiensis* Use in Agriculture: A Molecular Perspective, *Biological Reviews*, Vol.71, No.4, (November 1996), pp. 561–636, ISSN 1464-7931

Capalbo, D.M.F.; Valicente, F.H.; Moraes, I.O. & Pelizer, L.H. (2001) Solid-state Fermentation of *Bacillus thuringiensis tolworthi* to Control Fall Armyworm in Maize, *Electronic Journal of Biotechnology*, Vol.4, No.2, (August 2001), pp. 112-115, ISSN 0717-3458

Charles, J.F.; Silva-Filha, M.H.; Nielsen-Leroux, C.; Humphreys, M.J. & Berry, C. (1997) Binding of the 51- and 42-kDa Individual Components From the *Bacillus sphaericus* Crystal Toxin to Mosquito Larval Midgut Membranes From *Culex* and *Anopheles* sp. (Diptera: Culicidae). *FEMS Microbiology Letters*, Vol.156, No.1, (November 1997), pp. 153-159, ISSN 1574-6968

Couch, T.L. (2000). Industrial Fermentation and Formulation of Entomopathogenic Bacteria. In: *Entomopathogenic Bacteria: From Laboratory to Field Application*, J.F. Charles, A. Delécluse, C. Nielsen-LeRoux (Eds.), 297-314, Kluwer Academic Publishers, ISBN 0-7923-6523-2, Dordrecht, The Netherlands

Dias, J.M.C.S. (1992) Produção e Utilização de Bioinseticidas Bacterianos, *Pesquisa Agropecuária Brasileira*, Vol.27, (April 1992), pp. 59-76, ISSN 0100-204X

El-Bendary, M.A. (2010) Production of Mosquitocidal *Bacillus sphaericus* by Solid State Fermentation Using Agricultural Wastes, *World Journal of Microbiology and Biotechnology*, Vol.26, No.1, (January 2010) pp. 153-159, ISSN 0959-3993

Flores, E.R.; Pérez, F. & de la Torre, M. (1997) Scale-up of *Bacillus thuringiensis* Fermentation Based on Oxygen Transfer, *Journal of Fermentation and Bioengineering*, Vol.83, No.6, pp. 561-564, ISSN 0922-338X

Goldberg, L.J. & Margalit, J.A. (1977) A Bacterial Spore Demonstrating Rapid Larvicidal Activity Against *Anopheles sergentii*, *Uranotaenia unguiculsts*, *Culex univiattus*, *Aedes aegypti* and *Culex pipiens*, *Mosquito News*, Vol.37, No.3, (September 1977), pp. 355-358, ISSN 0027-142X

Honée, G. & Visser, B. (1993) The Mode of Action of *Bacillus thuringiensis* Crystal Proteins, *Entomologia Experimentalis et Applicata*, Vol.69, No.2, (November 1993), pp. 145–155, ISSN 1570-7458

Hongyu, Z.; Ziniu, Y. & Wangxi, D. (2000) Isolation, Distribution and Toxicity of *Bacillus thuringiensis* from Warehouses in China, *Crop Protection*, Vol.19, No.7, (August 2000), pp. 449-454, ISSN 0261-2194

Hsu, Y.L. & Wu, W.T. (2002) A Novel Approach for Scaling-up a Fermentation System. *Biochemical Engineering Journal*, Vol.11, No.2-3, (September 2002), pp. 123–130, ISSN 1369-703X

Huang, T.K., , Wang, P.M. & Wu, W.T. (2001) Cultivation of *Bacillus thuringiensis* in an Airlift Reactor with Wire Mesh Draft Tubes, *Biochemical Engineering Journal*, Vol. 7, No. 1, (January 2001), pp. 35-39, ISSN 1369-703X

Humphrey, A (1998) Shake Flasks to Fermentor: What Have We Learned? *Biotechnology Progress*, Vol.14, No.1, (January 1998), pp. 3–7, ISSN 1520-6033

Jing-Wen, Z.; Ya-Fei, C.; Zheng-Hong, X.; Zi-Niu, Y. & Shou-Wen, C. (2007) Production of thuringiensin by Fed-batch Culture of *Bacillus thuringiensis* subsp. *darmstadiensis* 032 with an Improved pH-control Glucose Feeding Strategy, *Process Biochemistry*, Vol.42, No.1, (January 2007), pp. 52-56, ISSN: 1359-5113

Jong, J.Z.; Wu, W.T. & Tzeng, Y.M. (1994) pH Control for Fed-batch Culture of *Bacillus thuringiensis*, *Biotechnology Techniques,* Vol.8, No.7, (July 1994), pp. 483–486, ISSN 0951-208X

Jong, J.Z.; Hsiun, D.Y.; Wu, W.T.; Tzeng, Y.M. (1995) Fed-batch Culture of *Bacillus thuringiensis* for thuringiensin Production in a Tower Type Bioreactor, *Biotechnology and Bioengineering*, Vol.48, No.3, (November 1995), pp. 207–213, ISSN1097-0290

Ju, L.K. & Chase, G.G. (1992) Improved Scale-up Strategies of Bioreactors. *Bioprocess and Biosystems Engineering*, Vol.8, No.1-2, (October 1992), pp. 49–53, ISSN 1615-7591

Kellen, W.R.; Clark, T.B.; Lindegren, J.E.; Ho, B.C.; Rogoff, M.H. & Singer, S. (1965) *Bacillus sphaericus* Neide as a Pathogen of Mosquitoes, *Journal of Invertebrate Pathology*, Vol.7, No.4, (December 1965), pp. 442-448, ISSN 0022-2011

Kumar, P.A.; Sharma, R.P. & Malik, V.S. (1996) the Insecticidal Proteins of *Bacillus thuringiensis*, *Advances in Applied Microbiology*, Vol.42, pp. 1-43, ISBN 9780120026425

Luna, C.L.; Silva, G.R. & Rios, E.M.M.M. (2004) *Bacillus thuringiensis* var. *israelensis* Production Involving Re-use of the Supernatant, *Biotechnology Letters*, Vol.26, No.2, (January 2004), pp. 143–145, ISSN 1573-6776

Luna, C.L.; Rios, E.M.M. & Lopes, C.E. (2001) On the Settling of *Bacillus sphaericus* Spores by pH Adjustment, *Biotechnology Letters*, Vol.23, No.12, (June 2001), pp. 1011-1013, ISSN 1573-6776

Luna, C.L.; Lopes, C.E. & Massarani, G. (2003) Recovery of *Bacillus sphaericus* 2362 Spores from Growth Medium by Flocculation/Sedimentation, *Bioprocess and Biosystems Engineering*, Vol.25, No.4, (January 2003), pp. 213–216, ISSN 1615-7605

Luna, C.L.; Lopes, C.E. & Massarani, G. (2005) Recovery of *Bacillus sphaericus* Spores by Flocculation/Sedimentation and Flotation, *Brazilian Archives of Biology and Technology*, Vol.48, (June 2005), pp. 61-70, ISSN 1516-8913

Luna-Finkler, C.L. & Finkler, L. (2008) Production of Concentrates of Bacterial Bio-insecticide *Bacillus thuringiensis* var. *israelensis* by Flocculation/Sedimentation, *Acta Tropica*, Vol.107, No.2, (August 2008), pp. 134–138, ISSN 0001-706X

Mignone, C.F. & Avignone-Rossa, C. (1996) Analysis of Glucose Carbon Fluxes in Continuous Cultures of *Bacillus thuringiensis*, *Applied Microbiology and Biotechnology*, Vol.46, No.1, (August 1996), pp. 78–84, ISSN 0175-7598

Navon, A. (2000) *Bacillus thuringiensis* Insecticides in Crop Protection – Reality and Prospects, *Crop Protection*, Vol.19, No.8-10, (September 2000), pp. 669-676, ISSN 0261-2194

Pearson, D. & Ward, O. P. (1988) Effect of Culture Conditions on Growth and Sporulation of *Bacillus thuringiensis* subsp. *israelensis* and Development of Media for Production of the Protein Crystal Endotoxin, *Biotechnology Letters*, Vol.10, No.7, (July 1988), pp. 451-456, ISSN 1573-6776

Prabakaran, G. & Balaraman, K. (2006) Development of a Cost-effective Medium for the Large Scale Production of *Bacillus thuringiensis* var *israelensis*, *Biological Control,* Vol.36, No.3, (March 2006), pp. 288-292, ISSN: 1049-9644

Rivera, D.; Margaritis, A. & Lasa, H. (1999) A Sporulation Kinetic Model for Batch Growth of *B. thuringiensis*, *The Canadian Journal of Chemical Engineering*, Vol.77, No.5, (October 1999), pp. 903–910, ISSN 1939-019X

Rukmini, V.; Reddy, C.Y. & Venkateswerlu, G. (2000) *Bacillus thuringiensis* Crystal δ-endotoxin: Role of Proteases in the Conversion of Protoxin to Toxin, *Biochimie*, Vol.82, No.2, (February 2000), pp. 109−116, ISSN 0300-9084

Russel, B.L.; Jelley, S.C. & Yousten, A.A. (1989) Carbohydrate Metabolism in the Mosquito Pathogen *Bacillus sphaericus* 2362, *Applied and Environmental Microbiology*, Vol.55, No.2, (February 1989), pp. 294-297, ISSN 0099-2240

Salama, H.S.; Foda, M.S.; Dulmage, H.T. & El-Sharaby, A. (1983) Novel Fermentation Media for Production of δ-endotoxins from *Bacillus thuringiensis*, *Journal of Invertebrate Pathology*, Vol.41, No.1, (January 1983), pp. 8-19, ISSN: 0022-2011

Selinger, L.B.; Dawson, P.S.S. & Khachatourians, G.G. (1988) Behavior of *Bacillus thuringiensis* var. *kurstaki* Under Continuous Phased Cultivation in a Cyclone Fermentor, *Applied Microbiology and Biotechnology*, Vol.28, No.3, (May 1988), pp. 247-253, ISSN 0175-7598

Saksinchai, S.; Suphantharika, M. & Verduyn, C. (2001) Application of a Simple Yeast Extract from Spent Brewer's Yeast for Growth and Sporulation of *Bacillus thuringiensis* subsp. *kurstaki*: a Physiological Study, *World Journal of Microbiology and Biotechnology*, Vol.17, No.3, (April 2001), pp. 307-316, ISSN: 0959-3993

Silva, M.; Furigo Junior, A.; Furlan, S.A. & Souza, O. (2011) Production of Bio-inseticide *Bacillus thuringiensis* var. *israelensis* in Semicontinuous Processes Combined With Batch Processes for Sporulation, *Brazilian Archives of Biology and Technology*, Vol.54, No.1, (January/February 2011), pp. 45-52, ISSN 1516-8913

Singer, S. (1973) Insecticidal Activity of Recent Bacterial Isolates and Their Toxins Against Mosquito Larvae, *Nature*, Vol.244, No.5411, (July 1973), pp. 110-110, ISSN 0028-0836

Smith, R.A. (1982) Effect of Strain and Medium Variation on Mosquito Toxin Production by *Bacillus thuringiensis* serovar. *israelensis*, *Canadian Journal Microbiology*, Vol.28, No.9, (September 1982), pp. 1089-1092, ISSN 0008-4166

Vimala Devi, P.S.; Ravindera, T. & Jaideva, C. (2005) Cost-effective Production of *Bacillus thuringiensis* by Solid-state Fermentation, *Journal of Invertebrate Pathology*, Vol.88, No.2, (February 2005), pp. 163-168, ISSN 0022-2011

Vontersch, M.A.; Slatin, S.L.; Kulesza, C.A. & Eglish, G.H. (1994) Membrane Permeabilising Activities of *Bacillus thuringiensis* Cry IIIB2 and Cry IIIB2 Domain I Peptide, *Applied and Environmental Microbiology*, Vol.60, No.10, (October 1994), pp. 3711–3717, ISSN 0099-2240

Vu, K.D.; Tyagi, R.D.; Valéro J.R. & Surampalli, R.Y. (2010) Batch and Fed-batch Fermentation of *Bacillus thuringiensis* Using Starch Industry Wastewater as Fermentation Substrate, *Bioprocess Biosystem Engineering,* Vol.33, No.6, (August 2010), pp.691-700, ISSN 1615-7591

Weiser, J.A. (1984) A Mosquito-Virulent *Bacillus sphaericus* in Adult *Simulium damnosum* From Northen Nigeria, *Zentralbatt fur Mikrobiologie*, Vol.139, No.1, (January 1984), pp. 57-60, ISSN 0232-4393

Wu, W.T.; Hsu, Y.L.; Ko, Y.F. & Yao, L.L. (2002) Effect of Shear Stress on Cultivation of *Bacillus thuringiensis* for thuringiensin production, *Applied Microbiology and Biotechnology*, Vol.58, No.2, (January 2002), pp. 175-177, ISSN 0175-7598

Yang, X.M. & Wang, S.S. (1998) Development of *Bacillus thuringiensis* Fermentation and Process Control from a Practical Perspective, *Biotechnology Applied Biochemistry*, Vol.28, No.2, (October 1998), pp. 95–98, ISSN 1470-8744

Yezza, A.; Tyagi, R.D.; Valero, J.R.; Surampalli, R.Y.; Smith, J. (2004) Scale-up of Biopesticide Production Processes Using Wastewater Sludge as a Raw Material, *Journal of Industrial Microbiology and Biotechnology*, Vol. 31, No.12 (November 2004), pp. 545–552, ISSN 1367-5435

Yousten, A.A. & Wallis, D.A. (1987) Batch and Continuous Culture of the Mosquito Larval Toxin of *Bacillus sphaericus* 2362, *Journal of Industrial Microbiology & Biotechnology*, Vol.2, No.5, (December 1987), pp. 277-283, ISSN 1367-5435

28

Entomopathogenic Nematodes (Nematoda: Rhabditida) in Slovenia: From Tabula Rasa to Implementation into Crop Production Systems

Žiga Laznik and Stanislav Trdan
University of Ljubljana, Biotechnical Faculty, Dept. of Agronomy, Chair of Phytomedicine, Agricultural Engineering, Crop Production, Pasture and Grassland Management, Ljubljana

1. Introduction

The interest in the use of entomopathogenic nematodes as biological pest control agents has increased exponentially over the past decades. A hundred different laboratories explore these nematodes and their bacterial symbionts in more than 60 countries from every inhabited continent. Despite research breadth that extends from molecular biology to field ecology, the discipline is unified by common interest in biological control. Thirty years ago, the idea of using nematodes to control pest populations was vague promise held by the handful of researchers working with these obscure insect parasites. Today, they are no longer a laboratory curiosity but have begun to gain acceptance as environmentally benign alternatives to chemical insecticides. The entomopathogenic nematodes have proven particularly successful and are now commercially mass-produced in six of the seven continents to treat pest problems in agriculture, horticulture and human husbandry. The ease of mass production and exemption from registration requirenments are the two major reasons for early interest in the commercial developments of entomopathogenic nematodes. However, demonstrations of practical use, particularly in Europe and North America and subsuquently in Japan, China and Australia, spurred developments across the world that have led to the availability of nematodes against pests that were once thought impossible to control.

Studies of entomopathogenic nematodes (EPNs) are nonetheless in many countries of the world limited to laboratory work. The reason for this lies in the fact that nematodes are in such areas still regarded as the so-called alien species, since their presence has not been confirmed in natural environment. The first studies of EPNs in Slovenia began within the project L4-6477-0481-04 in 2004. In Slovenia the Rules on biological plant protection (2006) prohibit introduction of alien species into natural environment. Since until 2007 EPNs were in Slovenia considered as foreign species, all studies had been limited to laboratory experiments. Because we wanted to implement their use in food production in Slovenia, we decided to study the presence of EPNs also in our soil. After discovering these biological agents, Slovenia became one of the countries where the use of nematodes as means of biological protection is sanctioned by law also for outdoors application.

2. Entomopathogenic nematodes as biocontrol agents

2.1 Environmentally acceptable food production

Integrated plant protection should not and cannot be equated with organic food production – which is according to some consumers the only way to produce healthy food. Consumers must be given opportunities to choose between organically produced food and food produced by other technologies, which imply less risk and lower costs. The state control system should ensure that food produced with the use of some synthetic substances is equally unblemished and healthy (Milevoj, 2002).

The most misgivings among consumers are caused by the use of products which protect plants against illnesses, harmful pests and weeds. The first principle of integrated protection is that food producers should use plant protection products only when other technological measures are no longer sufficient. In doing this only less toxic and faster degradable preparations are allowed. One of environmentally acceptable measures is also biological suppression of harmful pests on cultivated plants. Biological control is according to the definition of the International Organization for Biological and Integrated Control of Noxious Animals and Plants based on the use of living organisms (biological agents) or their products in order to prevent or diminish losses or damage caused to plants by harmful organisms. Biological control encompasses the so-called »beneficial organisms«. These are entomophagous insects which feed on phytophagous insects, mites and other Arthropoda, entomopathogenic microorganisms (fungi, bacteria, viruses, protozoa), which cause illnesses in pests harmful to plants and antagonists in the narrower sense (fungi, bacteria) – controlling phytopathogenic microorganisms (Milevoj, 1998).

The use of biological preparations requires additional knowledge from users, as well as a keener environmental awareness. Biologically based preparations are ecologically more appropriate, while their activity is more specific, their formulation, application and precision in timing of treatments are more important. However, they are often not as efficient as chemical preparations (Milevoj, 2002).

2.2 Entomopathogenic nematodes

Entomopathogenic nematodes (EPNs) of the families Steinernematidae and Heterorhabditidae are lethal pathogens of insects. These pathogens contribute to the regulation of natural populations of insects, but the main interest in them is an inundatively applied biocontrol agent (Kaya and Gaugler, 1993). Their success in this role can be attributed to the unique partnership between a host-seeking nematode and a lethal insect-pathogenic bacterium. Because of their biocontrol potential, considerable attention has been directed over the past few decades to genus *Heterorhabditis* and *Steinernema* and their respectative bacterial partners, *Photorhabdus* and *Xenorhabdus* (Forst and Clarke, 2002).

Although heterorhabditids and steinernematids are not closely related (Poinar, 1993), they have many features in common. These similarities, including their association with insect-pathogenic bacteria, are presumed to have arison through convergent evolution (Poinar, 1993). In both *Steinernema* and *Heterorhabditis* there is a single free-living stage, the infective juvenile (IJ), that carries in its gut bacteria of the genus *Xenorhabdus* and *Photorhabdus*, respectively (Boemare *et al*., 1993). On encountering a suitable insect, the IJ enters through the mouth, anus or spiracles and makes its way to the haemocoel (Kaya and Gaugler, 1993). Some species may also penetrate through the intersegmental membranes of the insect cuticle. In the haemocoel, the IJ releases cells of its bacterial symbiont from its intestine.

Bacteria multiply rapidly in haemolymph and produce toxins and other secondary metabolites, which contribute to the weakening of the host's defence mechanism. The host attacked by entomopathogenic nematodes usually dies because of poisoning or failure of certain organs in 24 to 72 hours after the infection (Forst and Clarke, 2002). Two developmental cycles thus occur in the host – that of nematodes and that of bacteria. The first generation nematodes pass into the second generation. After larvae cast off the fourth sheath and in the adult period, nematodes pass into the third generation, which thrives in the host as long as there is food. The host is by then already dead – killed by the toxins secreted by bacteria. The third generation nematodes are thus already saprophagic. Bacteria also produce such toxins (3.5 dihydroxy-4-isopropyl-stilben) which deter other micro organisms from settling in the carcass. When the developmental cycle is finished, nematodes leave the parts of carcasses which have not decomposed and they return into the ground. Nematodes cannot develop without a host (an insect) (Kaya and Gaugler 1993), without it they live in the ground for only a very brief period.

The importance of EPNs and biological plant protection against harmful organisms was first established in the USA in the thirties of the previous century. In 1923 Glaser and Fox discovered a nematode which attacked and caused death of the beetle *Popillia japonica* Newman (Glaser and Farell, 1935). Glaser introduced a method of growing EPNs »*in vitro*«. With such nematodes he was in 1939 carried out the first field experiment in New Jersey to suppress *Popillia japonica* (Kaya and Gaugler, 1993).

When entomopathogenic nematodes were first discovered, the hypothesis was proposed that nematodes alone cause death of the attacked insects. In 1937 Bovien first mentioned the possibility for the existence of symbiotic bacteria which live with entomopathogenic nematodes in a mutualist relationship. His hypothesis was in 1955 confirmed by Dutky and Weiser (Weiser, 1955). Boemare in 1982 proved that nematodes from the genus *Steinernema* produce toxic substances which negatively influence the immune system of infected insects and can themselves – without the presence of symbiotic bacteria – cause death of the host. For entomopathogenic nematodes from the genus *Heterorhabditis* it has not yet been established that they can alone produce toxic substances which would diminish the vitality of infected insects (Kaya and Gaugler, 1993).

The use of entomopathogenic nematodes in biological plant protection was some years ago still traditionally connected with suppressing soil-inhabiting insect pests (Ishibashi and Choi, 1991). The research results in the last two decades indicate they have also potential to suppress above-ground insect pests, but only in certain conditions (Trdan *et al.*, 2008; Laznik *et al.*, 2011). Lesser efficiency of entomopathogenic nematodes in suppression of above-ground insect pests is primarily due to inappropriate (insufficient) moisture, exposure to thermal extremes and ultraviolet radiation. These factors are of crucial importance for the survival of nematodes (Kaya and Gaguler, 1993). For this reason nematodes are less efficient against above-ground insect pests outdoors, though the previous laboratory tests showed much higher efficiency (Laznik *et al.,* 2010c).

To lay nematodes on plants we can use equipment which is intended for spraying plant protection products, manuring or irrigation. For this purpose backpack manual or tractor sprayers, sprinklers and also planes are suitable. Infective larvae can be passed through spray tubes with diameter of at least 500 µm, capable to withstand pressure up to 2000 kPa (Kaya and Gaugler, 1993).

EPN infective juveniles (IJs) can tolerate short-term exposure (2-24 hrs.) to many chemical and biological insecticides, fungicides, herbicides, fertilizers, and growth regulators, and can

thus be tank-mixed and applied together (Koppenhöfer *et al.* 2002; De Nardo and Grewal 2003). Nematode-chemicals combinations in tank-mixes could offer a cost-effective alternative to foliar integrated pest management (IPM) systems.
Due to the sensibility of nematodes to ultraviolet radiation, they have to be applied to plants in the evening, early in the morning or in a cloudy weather, when the radiation is not so intense (Kaya and Gaugler, 1993). Nematode survival and efficacy on foliage has also been shown to be enhanced to varying degrees by addition of various adjuvants to the spray mixture, which have antidesiccant (e.g. glycerol, various polymers) or UV-protective (brighteners) actions (Grewal, 2002) although more needs to be done to enhance post-application survival. The greatest potential for using EPNs against foliar pests is almost certainly in IPM programmes, in conjunction with other biocontrol agents (Sher and Parella, 1999) or selective chemicals (Rovesti and Deseo, 1990).
EPNs are considered exceptionally safe biological agents (Akhurst and Smith, 2002). Because their activity is specific, their environmental risk is considerably lower than that of chemical agents for plant protection. Since the first use of EPNs for suppressing *Popillia japonica* Newman in the USA (Glaser and Farrell, 1935) until now, no case of environmental damage due to these biological agents has been documented. The use of nematodes is safe for users. EPNs and their bacteria are not harmful for mammals and plants (Akhurst and Smith, 2002).

3. Entomopathogenic nematodes as an exotic organisms in Slovenia

The basis for regulating biological control of plants in Slovenia as an appropriate way to control harmful organisms in agriculture and forestry was provided by the Plant Health Act in 2001, which divided the management of this field between the Phytosanitary Administration of the Republic of Slovenia and the Ministry of the Environment and Spatial Planning, competent for the preservation of nature. The field is in more detail regulated by the Rules on biological plant protection (Official Gazette no. 45/06), which came into force on the 13th of May 2006. The rules regulate introduction, cultivation and use of beneficial organisms, such as living natural enemies, antagonists, competitors or their products, and other organisms which are able to reproduce by themselves, including those which are packed or formulated as commercial products for biological protection of plants. The Rules' provisions do not apply to introduction and use of micro organisms which are covered by the regulations in the field of plant protection products. Beneficial organisms which can be used for biological protection of plants may be autochthonous (the species naturally present in a certain ecosystem) or allochthonous (the species which were introduced by man and which were before that not present in a certain ecosystem). The introduction of allochthonous organisms into nature requires special caution, so the application for introduction should be accompanied by risk assessment for nature – according to the regulation on risk assessment for nature. Only those allochthonous species which are on the list published by the Minister, in consensus with the Minister responsible for the preservation of nature, can be used for biological protection of plants in greenhouses and outdoors.
As already mentioned, the first studies of EPNs in Slovenia began in 2004. Until 2007 EPNs were considered allochthonous organisms, all our studies had been thus limited to laboratory experiments. The goal of the said studies was to investigate the effects different species of nematodes at different temperatures and concentrations of suspensions have on different species of harmful insects (Laznik and Trdan, 2008ab). The novelty in our approach

was the selection of harmful pests, as we wanted to investigate the effects of EPNs on above-ground pests. In Slovenia this approach was for the first time used by Simona Perme (2005), who under the mentorship of Prof. Dr. Stanislav Trdan in her Master thesis studied the effects of EPNs on three species of harmful insects which cause damage by sucking or biting above-the-ground parts of plants.
The laboratory experiments in Slovenia thus tested the efficiency of EPNs against Colorado potato beetle (*Leptinotarsa decemlineata* [Say]) (Perme, 2005), greenhouse whitefly (*Trialeurodes vaporariorum* [Westwood]) (Perme, 2005), western flower thrips (*Frankliniella occidentalis* [Pergande]) (Perme, 2005), sawtoothed grain beetle (*Oryzaephilus surinamensis* [L.]) (Trdan *et al*., 2006), granary weevil (*Sitophilus granarius* [L.]) (Trdan *et al*., 2006), *Hercinothrips femoralis* (Reuter) (Trdan *et al*., 2007a), flea beetles (*Phyllotreta* spp.) (Trdan *et al*., 2008), cabbage stink bug (*Eurydema ventrale* Kolenati) (Zupančič, 2008), common cockchafer (*Melolontha melolontha* [L.]) (Laznik *et al*., 2009d), and cereal leaf beetle (*Oulema melanopus* [L.]) (Laznik *et al*., 2010b). These experiments confirmed the facts that had been already established, namely that EPNs are in optimal circumstances exceptionally efficient agents for suppressing harmful insects (Laznik and Trdan, 2008ab). Since we wanted to implement their use in food production in Slovenia, we decided to find out to what extent they are present in our soil.

4. Establishing the presence of entomopathogenic nematodes in Slovenia

4.1 Recovery from soil

Isolation of entomopathogenic nematodes from soil can be accomplished by standard extraction techniques for soil nematodes. Numerous nematodes are recovered, so entomopathogenic ones need to be separated and identified, a laborious procedure resulting in the processing of fewer samples compared with other methods (Kaya and Gaugler, 1993). Also, identification of the juveniles requires particular taxonomic expertise so only a few scientists are capable of the task. The alternative is to use a *Galleria* baiting technique which was used also in our experiments.

4.1.1 *Galleria* baiting technique and White trap method

The baiting technique with larvae of the greater wax moth, *Galleria melonella* (L.), is the method most commonly used for recovering infective-stage juveniles of entomopathogenic nematodes from soil (Bedding and Akhurst, 1975). The efficiency of this method had been increased using several consecutive baiting rounds (Fan and Hominick, 1991) and by baiting at two temperatures (Mráček and Bečvář, 2000).
The soil samples, five from each sampling place, were taken. Each soil sample (approximately 1 kg) was a composite of 3 random subsamples taken at a depth of 3 – 15 cm in an area of 20 m^2. The samples were taken at least 100 m apart at each site. The samples were placed in polyethylene bags to prevent water loss and were kept in coolers (ca. 15 ^oC) during transit to the laboratory. We put a living larva of a greater wax moth into an Eppendorf tube which had been pierced. In each soil sample 5 insect host Eppendorf tubes were placed. Mortality of *G. melonella* was assessed after 5 days. Dead *Galleria* larvae were used for culturing on a water trap to obtain infective juveniles. The dead larvae of a greater wax moth were dried at room temperature for 10 days. Dead insects were incubated individually in modified »White traps« (White, 1929) which consisted of a glass Petri dish (9 cm in a diameter) filled with destilated water to a depth of 0.5 cm. The bottom of an inverted

Petri dish (3 cm in a diameter) was placed in the bigger Petri dish. A sheet of filter paper was placed on the smaller Petri dish allowing the edge of the paper to come in a contact with the destilated water. The dead larvae were placed on the filter paper and incubated at room temperature until all the nematode progeny had emerged and moved down into the water of the bigger Petri dish. Following procedure contained the use of centrifuge and 5 % concetration of sodium hypoclorate. The aim of this process was to get infective juveniles from the suspension. With the received suspension we infected artificialy larvae of greater wax moth again. The harvested infective juveniles (IJs) were stored at 4 °C in distilled water (Laznik *et al*., 2008, 2009abc).

4.1.2 Molecular characterization

To confirm the identification of the isolated nematodes harvested from the larvae of the greater wax moth, a molecular characterization was conducted. Genomic DNA was extracted from individual nematodes and PCR was performed to multiply the ITS region using the primers TW81 and AB28, following Hominick *et al.* (1997). The PCR products were re-isolated from a 1 % TAE-buffered agarose gel using the E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, USA). The re-isolated sample was sequenced in the laboratory of the Agricultural Biotechnology Centre in Gödöllő, Hungary. The sequence was submitted to GenBank public database. The sample DNA sequence was compared to the sequences of the species *Steinernema* and *Heterorhabditis* using a BLAST search at the National Centre for Biotechnology Information (NCBI) web site (www.ncbi.nlm.nih.gov) (Laznik *et al*., 2008, 2009abc).

4.2. Distribution of entomopathogenic nematodes in Slovenia

Between the years 2006 and 2009 we analysed 570 soil samples from 114 different locations in Slovenia. The samples has been taken especially in areas that are considered suitable habitats (e.g. sandy soils, cultivated fields, grasslands, forests) for the presence of steinernematids and heterorhabditids. The presence of EPNs was confirmed in 31 samples, which is 5.4 % of the total number of the analysed soil samples (Laznik *et al*., 2008, 2009abc).

4.2.1 *Steinernema affine* Bovien

In the year 2007 we recorded the species *Steinernema affine* Bovien (Laznik *et al*., 2008) of the Steinernematidae family, which is in the intermedium group of nematodes (Bovien, 1937). It lives in symbiosis with the bacteria *Xenorhabdus bovienii* Akhurst (Poinar, 1988). The said organism was first found in 1937, while its usefulness in biological plant protection was discovered a few years ago (Willmott *et al*., 2002). This species often appears in soils where plants of the family Brassicaceae are cultivated (Nielsen and Philipsen, 2004). Cabbage was cultivated also in the field located not far from Staro selo near Kobarid, where we confirmed the presence of the said nematodes. *Steinernema affine* is known to be exceptionally efficient biological agent for suppressing cabbage root fly (*Delia radicum* [L.]) (Willmott *et al*., 2002; Nielsen and Philipsen, 2004). Cabbage is in Slovenia the most widely cultivated vegetable (24.1 % of all fields or 871 ha) (the Statistical Office of the Republic of Slovenia, 2005), which makes the said plant from the family Brassicaceae even more useful.

4.2.2 *Steinernema feltiae* (Filipjev)

The most frequently (15 positive samples) encountered species of EPNs in Slovenia was *Steinernema feltiae* (Filipjev) (accesion no: EU914855; http://blast.ncbi.nlm.nih.gov/Blast.cgi)

(Laznik *et al*., 2009a). In Slovenia it was found in Notranjska, Dolenjska and Prekmurje. The said species is in the so-called »feltiae group«, in which the length of the IJ ranges from 700 to 1000 µm. *S. feltiae* lives in symbiosis with the bacteria *Xenorhabdus bovienii* Akhurst (Poinar, 1988), and was first discovered as late as in 1934. Its usefulness in biological control is well known (Trdan *et al*., 2006). Some researchers report that *S. feltiae*, S. *intermedium* and *S. affine* are likely to appear on cultivated land (Sturhan, 1996). In our study we came to a similar conclusion only in one of the positive samples (the strain B30, which was found on a field near Cerknica), while all other strains were found on uncultivated areas (forests, meadows) (Laznik *et al*., 2009a). In Europe *S. feltiae* has been so far confirmed in 24 countries and is considered one of the most widely spread species of EPNs (Hominick, 2002). After our finding the said species became autochthonous in Slovenia and can be used also outdoors to suppress harmful insects (Decision ..., 2008a).

4.2.3 *Steinernema carpocapsae* Weiser

The second most spread species of EPNs in Slovenia (confirmed in 12 soil samples) was *Steinernema carpocapsae* Weiser (accesion no: EU914854; http://blast.ncbi.nlm.nih.gov /Blast.cgi) (Laznik *et al*., 2008). The species belong to the so-called »carpocapsae group«, IJs of these are below 600 µm and live in symbios with the bacteria *Xenorhabdus nematophila* Poinar & Thomas (Akhurst, 1980). It was first found in 1955, in Europe is present in 15 countries (Hominick, 2002). Since it can be relatively easily multiplied and can endure several months in total desiccation, it is massively used in plant protection (Kaya and Gaugler, 1993). It acquires hosts by passive ambush and is for this reason used for suppressing more mobile insect species (Campbell *et al*., 2003). Its optimal temperature interval is between 22 and 28 °C. The abovementioned species became autochthonous in Slovenia on the 1st of September 2008 (Conclusion ..., 2008b).

4.2.4 *Steinernema kraussei* Steiner

In the Gorenjska region (the north-west region of Slovenia) we discovered in two samples also *Steinernema kraussei* Steiner (accession no.: EU914856; http://blast.ncbi.nlm.nih.gov /Blast.cgi) (Laznik *et al*., 2009b), which is characteristically active also at slightly lower temperatures (between 6 and 10 °C). The said species belongs to the so-called »feltiae group« and lives in symbiosis with the bacteria *Xenorhabdus bovienii* Akhurst (Fischer-Le Saux *et al*., 1998). *S. kraussei* was the first species to be found in the world, discovered in 1923 (Glaser and Fox, 1930). The species served in the first field experiment (in 1939) suppressing the beetle *Popillia japonica* Newman. Many researchers confirmed high efficiency of this species at low tempetrature (Long *et al*., 2000; Willmott *et al*., 2002), what is of great importance for suppression of harmful insects outdoors since thermal extremes, UV radiation levels and lack of moisture importantly influence EPNs activity (Kaya and Gaugler, 1993). This species also was in Slovenia listed as autochthonous organism for biological protection of plants (Conclusion ..., 2009a).

4.2.5 *Heterorhbaditis bacteriophora* Poinar

In August 2008 in the Dravograd region (the north-east Slovenia) the presence of *Heterorhabditis bacteriophora* Poinar was confirmed (accession no.: FJ477060; http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Laznik *et al*., 2009c). It is so far the only found nematode from the genus *Heterorhabditis* in Slovenia. In Europe 4 species from the said

genus have been discovered so far, the said species is in Europe present in 12 countries (Hominick, 2002). The infective larvae of *H. bacteriophora* range from 520 to 600 µm. The nematode acquires hosts actively by detecting vapourised substances (released CO_2) (O'Halloran in Burnell, 2002). The species has great potential in biological plant protection (Koppenhöfer *et al*., 2004; Grewal *et al*., 2005). In Slovenia the said species became autochthonous on the 15th of January 2009 (Conclusion ..., 2009b) on the basis of the application for changing the status of allochthonous organism submitted by the Biotechnical Faculty on the 6th of January 2009 to the Ministry of Agriculture, Food, and Forestry of the Republic of Slovenia – the Phytosanitary Administration of the Republic of Slovenia. The status of autochthonous species was in the same way acquired by the *S. feltiae*, *S. kraussei* in *S. carpocapsae*.

5. Laboratory experiments

5.1 *In vivo* production of the EPNs

For laboratory use and small-scale field testing, *in vivo* production of entomopathogenic nematodes (EPN) is the appropriate method. When it comes to commercial use of EPN at a larger scale for international markets, *in vitro* production is currently the only economically reasonable means to supply EPN at high quality and at reasonable costs. *In vivo* production method for culturing EPN in insect hosts have been reported by various authors (Flanders *et al*., 1996). These references essentially describe systems based on the White trap (White, 1929), which take advantage of the infective juvenile's (IJ) natural migration away from host cadaver upon emergence. The most common insect host used for in vivo production is the last instar of the greater wax moth (*Galleria melonella)*, because of its high susceptibility to most nematodes, ease in rearing, wide availability and ability to produce high yields (Ehlers, 2001). After 2-5 days, infected larvae of the greater wax moth are transferred to the White traps. Following harvest, concentration of nematodes can be accomplished by gravity settling (Flanders *et al*., 1996). Following procedure contained the use of centrifuge and 5 % concetration of sodium hypoclorate. The aim of this process was to get infective juveniles from the suspension.

5.2 Preparing the concentration of nematode suspension

We tested the efficacy of the EPNs in controlling different stages (larvae, adults) of a different insect species by exposing individuals to either 0, 250, 500, 1000, or 2000 IJ/individual. We determined the number of infective juveniles (IJs) in a previously (see above) prepared unknown concentration of nematode suspension by counting the number of such in droplets (5 µl x 5) and by diluting (adding M9 solution) or by concentrating (reduction to an adequate volume with the assistance of centrifugation). In this manner we obtained the selected concentrations of nematode suspensions (0, 2500, 5000, 10000, and 20000 IJ/ml). We used only infective juveniles which were less than 2 weeks old. During the experiment, which was repeated three times, we stored the infective juveniles at 4 °C (Laznik *et al*., 2010a).

5.3 Laboratory bioassay

We carried out the experiment according to the procedure described in the paper of Trdan *et al*., 2006. The following procedure was performed with a time interval in three replications. We placed tested insect species in glassy Petri dishes (diameter = 9 cm) with

each containing 10 individuals. Prior to this, we put filter paper into each Petri dish (the same diameter as the former Petri dish) and some additional food (specific for the tested insect). Each treatment in the experiment was repeated 10 times. The assigned nematode concentration was added to the filter paper with a pipette (1 ml). The Petri dishes were put in a rearing chamber (type: RK-900 CH, producer: Kambič Laboratory equipment, Semič, Slovenia) with a volume of 0.868 m^3 (width x height x depth = 1000 x 1400 x 620 mm). We tested the efficacy at different temperatures (15, 20, 25, and 30 °C) and at a relative humidity of 80 %. The number of dead individuals was determined 2, 4, 6, and 8 days after treatment. The dead individuals were dissected to determine if the nematodes were present. In such a manner we wanted to prove that the insects died due to the EPNs' activity.

5.4 Statistical analysis

A multifactor analysis of variance (ANOVA) was conducted to determine the differences in mortality rates (%) between tested insect species reared in different treatments. Before the analysis, the mean mortality was tested for the homogeneity of treatment variances. Mortality rate data were corrected for control mortality, using Abbott's formula (Abbott, 1925). The arcsine square-root was transformed before this analysis. A Student-Newman-Keuls multiple range test ($P \leq 0.05$) was used to separate mean differences among the parameters in all the treatments. For the last days after treatment (DAT) the values of LC_{50} and LC_{90} (the numbers of IJs/individual causing 50% and 90% mortality) were estimated, and the overall efficacy of the tested nematodes was determined from this estimates (Trdan *et al*., 2008; Laznik *et al*., 2010a) All statistical analyses were performed using Statgraphics Plus for Windows 4.0 (Manugistics, Rockville, MD, USA) and the figures were created with MS Office Excel 2003. The data were presented as untransformed means ± SE.

6. Greenhouse experiment

6.1 Greenhouse experiment design – testing EPNs activity against western flower thrips and greenhouse whitefly on greenhouse-grown cucumbers

Location, production of the seedlings, types of substrates, and experimental design is detailed described in the paper published by Trdan *et al*. 2007a. The glasshouse temperature at the time of the experiment was 26.0 - 45.5 °C (8:00 a.m. - 20:00 p.m.) and 14.0 - 25.0 °C (21.00 p.m. – 7:00 a.m.), while the relative humidity was 17.5 - 68.0 % (8:00 a.m. - 20:00 p.m.) and 55.0 - 90.0 % (21.00 p.m. – 7:00 a.m.). Four cucumber plants in each sleeve (sub-plot) were sprayed with a suspension of commercial EPN. A standard rate of 2500 IJ per ml of water was used. Four plants were sprayed with insecticide, and the four control plants were sprayed with water. Before spraying each suspension of nematodes, 0.05 % of the surfactant Nu-Film-17 (a.i. di-1-p-methene, 96 %; manufacturer: Lances Links SA, Geneva, Switzerland; supplier: Karsia Dutovlje d.o.o., Ljubljana, Slovenia) was added to the sprayer to enable the suspension to move more effectively across the leaf surface (Trdan *et al*. 2007a, Laznik *et al*., 2011).

Weekly applications thereafter (1, 2, 3, 4, and 5 weeks after first application [AFA]) of entomopathogenic nematodes suspension (at glasshouse temperature) were made by an injector hollow cone nozzle TVI 80 02 attached to motor operated backpack sprayer (Trdan et al. 2007a). Sprays with insecticide were applied at the same time of the day (early evening) using a backpack sprayer with an injector nozzle ID 90 02 (Trdan *et al*. 2007a). For more detailed information see Trdan *et al*., 2007a and Laznik *et al*., 2011.

6.2 Observations and evaluations

Evaluations were taken at three time-points during the growing season (3, 5 and 7 AFA). At each time-point, three randomly selected plants of about the same height in each sub-plot were assessed for the number of the tested insect. Three leaves (lower, middle and upper part) from each chosen plant were evaluated.

Cucumber fruits were harvested when their diameters were 3-4 cm. The yield was classified into three groups according to harvest time (up to 3 AFA, between 3 and 5 weeks AFA and 5 weeks AFA). The cucumber yield was determined in two ways: 1) mass of fruits per plant, and 2) number of fruits per plant (Trdan *et al.* 2007a). Data analyses was made according to Trdan *et al.* 2007a.

7. Field experiment

7.1 Field experiment design - testing EPNs activity against Colorado potato beetle in potato

Experimental field (45 x 11 m) was divided into four blocks, and in each there were six treatments: control (unsprayed), domestic strain of EPN low conc., domestic strain of EPN high conc., commercial strain of EPN low conc., commercial strain of EPN high conc., and insecticide. The size of each treatment parcel was 20.9 m^2 (5.5 x 3.8 m). Agri-technical measures are detailed desribed in Laznik *et al*. 2010c.

We applied EPNs using the backpack sprayer Solo 425. The jet stream nozzle number 04F110 with a pressure of 2 bars was used. Two concentrations of nematode suspension for the foliar application against Colorado potato beetle were chosen: low at 250.000 IJ m^{-2} and high at 500.000 IJ m^{-2}. The application of all treatments were repeated, but only with half the concentration of nematode suspension (125.000 IJ m^{-2} and 250.000 IJ m^{-2}). For both applications 0.05% of the surfactant Nu-Film-17 (a.s. di-1-p-methene, 96 %; manufacturer: Lances Links SA, Geneva, Switzerland; supplier: Karsia Dutovlje d.o.o., Ljubljana, Slovenia) was added to EPNs' suspension, enabling the suspension to move more effectively across the leaf surface. We observed the population dynamics of Colorado potato beetle on day of application – (0 DAT), 3, 10, 16, 19 and 26 days after treatment (DAT). A visual inspection was made on the five selected plants in each treatment and the different developmental stages of CPB were counted throughout the course of the experiment.

The potatoes were harvested with a back output machine with two rolling plates on 12 August 2008 and on 6 August 2009. On the day of harvest the tubers were classified with a special shaking device into three fractions: fraction 1 (tubers <4 cm), fraction 2 (tubers between 4 and 5 cm), and fraction 3 (tubers > 5 cm) and weighed them separately as well as together. Later we calculated this to the t ha^{-1}.

7.2 Preparation of the EPNs

Commercial product was purchased (Koppert B.V., Berkel en Rodenrijs, The Netherlands) through the importer Zeleni hit d.o.o. (Ljubljana, Slovenia). The domestic nematode isolate was produced in a mechanically stirred, internal loop bioreactor with an 8000 ml capacity (BR021, Inel Ltd., Budapest, Hungary). After sterilization, the bioreactor containing the P2 culture media (23g yeast extract, 12.5g dried egg yolk, 5g NaCl and 40 ml corn oil in 1000 ml water, Chavarria-Hernandez and de la Torre, 2001) was inoculated with 100 ml of overnight bacterial symbiont culture, isolated from infective juveniles of the domestic strain. The temperature was 20 °C, and the aeration rate was 1.0

vvm. After two days, when the oxygen consumption of the bacterial culture decreased, the bioreactor was inoculated with five million infective juveniles of the domestic strain in 500 ml of culture media. The nematodes were harvested 14 days after, when the total number of nematodes was 25.000 individuals per ml, and the ratio of infective juveniles was 95 %. The nematodes were centrifugated and washed twice with sterilized tap water, and were stored in sterile M9 solution (5 g NaCl, 3 g KH_2PO_4 and 6 g Na_2HPO_4 in 1000 ml water).

7.3 Statistical analysis

Differences in the numerous developmental stages of the insect (egg clusters, larvae, adults) between individual treatments, as well as differences in yield were analysed with the use of ANOVA. Prior to analysis, each variable was tested for homogeneity of variance, and the data found to be non-homogenous was transformed to log(Y) before ANOVA. Significant differences ($P \leq 0.05$) between mean values were identified using Student-Newman-Keuls's multiple range test. All statistical analyses were done using Statgraphics Plus for Windows 4.0 (Statistical Graphics Corp., Manugistics, Inc.). The data was presented as untransformed means ± SE (Laznik *et al.*, 2010c).

8. Activity of entomopathogenic nematodes against selected pests of ornamental plants and vegetables in greenhouses and domestic environment

8.1 *Hercinothrips femoralis* (Reuter)

In the laboratory experiment we studied the efficiency of *Steinernema feltiae* and *Heterorhabditis bacteriophora* for suppressing banded greenhouse thrips – *Hercinothrips femoralis* (Trdan *et al.*, 2007b). At foliar application of the suspension with the concentration 200 IJ/specimen both species were relatively efficient at suppressing larvae and adult specimens of banded greenhouse thrips, though they were markedly more successful in suppressing larvae. Both species of nematodes reached the highest efficiency at 25°C, while independently of temperature and nematodes species the average corrected mortality rates in larvae was 37.7%, in adult specimens only 15.4% (Trdan *et al.*, 2007b). In view of the previous research results (Chyzik *et al.*, 1996; Premachandra *et al.*, 2003) we expected different efficiency of both biological agents in suppression of banded greenhouse thrips. The unexpected lesser efficiency of *H. bacteriophora* can be in this experiment attributed to the specific strain of the researched nematodes. The same species of EPNs are isolated in different parts of the world, while the results of many studies show that different nematode races of the same species differ considerably in their efficiency to suppress harmful pests (Premachandra *et al.*, 2003). Chyzik *et al.* (1996) found out, among other things, that *H. bacteriophora* strain HP88 is very efficient in suppressing banded greenhouse thrips, while the other strain (IS5) of the same species is much less efficient. Similar results were produced also by some other studies (Premachandra *et al.*, 2003).

8.2 Western flower thrips (*Frankliniella occidentalis* [Pergande])

In a greenhouse experiment, the effectiveness of *Steinernema feltiae* (Filipjev) (Rhabditida: Steinernematidae) was compared with abamectin for the control of western flower thrips (*Frankliniella occidentalis* [Pergande], Thysanoptera, Thripidae), on slicer cucumbers. In a

period from mid June to end of August, cucumbers were grown in four different growth substrates: expanded perlite, expanded vermiculite, light expanded clay aggregate and peat. A suspension of entomopathogenic nematodes (2500 infective juveniles/ml) was applied to cucumber leaves nine times in one growing season, whilst insecticide at recommended dose (22.5 g a.i./ha) was used three times. A significant effect in the extent of pest damage to the leaves (assessed by a six grade scale) was determined only for type of suppression (nematodes, insecticide, and untreated control), but not type of growth substrate nor damage evaluation date (16 July, 3 August, and 23 August). The leaves of cucumbers treated with nematodes and insecticide were significantly less damaged than untreated plants, with damage never exceeding 10% of the leaf surface. Nevertheless, type of growth substrate showed a significant effect on the number of fruits as well as on the mean mass of fruits. Light expanded clay aggregate was seen to be the least appropriate growth substrate, whilst the other three substrates can all be recommended for cucumber growing. The mean mass of fruits was also significantly influenced by type of suppression, with the mean mass of fruits in treated plants being significantly higher (from 37 up to 51 %) than in untreated plants. The mean number of fruits per plant did not differ significantly between different types of pest suppression. Nine times spraying with nematodes and three times spraying with abamectin showed about the same efficacy against western flower thrips on greenhouse-grown slicer cucumbers (Trdan *et al*., 2007b).

8.3 Greenhouse whitefly (*Trialeurodes vaporariorum* [Westwood])

The greenhouse whitefly (*Trialeurodes vaporariorum* [Westwood]) (GWF) is an important polyphagous harmful pest on cultivated plants (Vet *et al*., 1980). Adult specimens and larvae are harmful because they feed on plant sap, excrete honeydew and carry certain plant viruses (Coffin and Coutts, 1995). Because of excessive and inexpedient use of insecticides the said species developed resistance to some active substances (Gorman *et al*., 2001), so biological protection represents one of the alternative solutions for protecting plants against the said harmful pests. Some previous studies have shown that foliar application EPNs is inefficient for suppressing the selected harmful pests (Hara *et al*., 1993), some recent studies, however, have shown that we can by appropriate applying and by optimising the method of EPNs application successfully suppress also above-ground pests (Trdan *et al*., 2007a; Laznik *et al*., 2010c). The results of our study (Table 1) have shown that adult specimens of *T. vaporariorum* are sensitive to the activity of EPNs (Laznik *et al*., 2011). We expect that satisfactory mortality rates of insects are the result of applying EPNs several times in a row. When applying EPNs several times in a row, one should of course take into account the economic aspect of food production (Athanassiou *et al*., 2010; Laznik *et al*., 2010c). The results of our study have shown that feeding of adult specimens of the greenhouse whitefly influences also the average mass of cucumbers (table 2) (Laznik *et al*., 2011). The mass was importantly influenced by the manner in which the cultivated cucumbers were protected, while different substrates were in this regard of no consequence. A related study came to similar conclusions (Tocucumbersu and Abe, 2005). The smallest yield of cucumbers was in our study found on expanded clay, which was confirmed also by several other related studies (Cantliffe *et al*., 2003; Trdan *et al*., 2007a).

year	Time point (weeks AFA)	Substrates				Control methods		
		Expanded perlite	Vermi-culite	Light expanded clay aggregate	Peat	Untreated	thia-methoxam	EPNs
2007	3	3.00 ± 0.47a	4.22 ± 0.48b	5.94 ± 1.63b	23.61 ± 7.71c	21.13 ± 5.83c	2.58 ± 0.38a	3.88 ± 0.66b
	5	25.89 ± 4.24a	56.56 ± 9.80b	41.33 ± 8.94b	83.94 ± 15.33c	96.46 ± 11.26c	34.58 ± 4.52b	24.75 ± 4.35a
	7	58.22 ± 16.21a	51.28 ± 16.32a	58.78 ± 8.08a	74.68 ± 13.18a	119.17 ± 11.77c	25.64 ± 5.27a	39.46 ± 7.38b
2008	1	34.33 ± 23.14a	38.56 ± 10.66ab	43.56 ± 21.41ab	74.78 ± 25.12b	89.00 ± 21.62b	36.25 ± 15.83a	18.17 ± 7.26a
	2	32.00 ± 14.10a	67.67 ± 28.22ab	109.22 ± 43.31b	74.56 ± 10.01b	128.50 ± 33.00b	51.25 ± 13.30a	32.83 ± 9.53a

* Means with the same letter in the same line are not significantly different at $P = 0.05$ (Student-Newman-Keuls's multiple range test).

Table 1. Mean number of GWF adults per plant in 2007 and 2008 at different control methods and growth substrates.

year	Time point (weeks AFA)	Substrates				Control methods		
		Expanded perlite	Vermi-culite	Light expanded clay aggregate	Peat	Untreated	thia-methoxam	EPNs
2007	3	217.60 ± 10.75a	312.52 ± 19.83c	260.12 ± 22.01b	261.73 ± 15.39b	229.91 ± 12.92a	276.73 ± 17.47b	276.88 ± 14.86b
	5	279.43 ± 17.73a	294.50 ± 15.36a	286.19 ± 26.22a	292.19 ± 19.52a	254.35 ± 11.83a	290.69 ± 14.79b	326.04 ± 20.74c
	7	300.07 ± 17.56a	282.86 ± 20.58a	287.08 ± 23.09a	322.11 ± 34.29a	242.53 ± 18.55a	285.48 ± 18.72b	340.59 ± 21.38c
2008	1	237.62 ± 9.47a	295.56 ± 8.75c	277.91 ± 16.27bc	262.72 ± 15.97b	228.48 ± 9.47a	288.60 ± 11.52b	288.1 ± 11.13b
	2	270.36 ± 12.45a	290.61 ± 8.71b	271.63 ± 15.69ab	265.00 ± 15.25a	211.51 ± 6.46a	310.69 ± 10.27b	301.68 ± 10.17b

* Means with the same letter in the same line are not significantly different at $P = 0.05$ (Student-Newman-Keuls's multiple range test).

Table 2. Mean mass of cucumbers (g) in 2007 and 2008 at different control methods and growth substrates.

9. Activity of entomopathogenic nematodes against selected field crop pests

9.1 Flea beetles (*Phyllotreta* spp.)

Although flea beetles (*Phyllotreta* spp., Coleoptera, Chrysomelidae) are among more important harmful pests for the family Brassicaceae, in Europe as well as on some other continents EPNs have so far not been used to suppress them (Trdan *et al*., 2008). In 2005, four entomopathogenic nematode species (*Steinernema feltiae*, *S. carpocapsae*, *Heterorhabditis bacteriophora*, and *H. megidis*) were tested under the laboratory conditions with the aim of

studying their activity in controlling adult flea beetles, *Phyllotreta* spp. Activity of the biological agents studied was determined at three different concentrations (200, 1000, and 2000 IJs per adult) and temperatures (15, 20, and 25°C). Mortality of the beetles was determined 2, 4, 6, and 8 days after treatment. Efficacy of the nematodes was higher at the 20 and 25°C than at 15°C. At 20°C, eight days after treatment the nematodes killed from 43.5% (*H. megidis* at the lowest concentration) up to 77% (*S. feltiae* at the lowest concentration) of the beetles. At 25°C, three nematode species (*S. feltiae, S. carpocapsae*, and *H. bacteriophora*) killed at least 74% of the beetles at both higher concentrations. *Steinernema feltiae* was the most efficient at the lowest temperature (LC_{50} = 483-1467 IJs/adult), and it is therefore considered to have the highest potential for the control of overwintered flea beetles (May) in the open as alternative to chemical insecticides. The nematodes *S. feltiae*, *S. carpocapsae* and *H. bacteriophora* proved themselves to be the most appropriate choice for controlling the adult flea beetles during warm summer months, when in Slovenia the pest occur in the highest numbers (Trdan *et al*., 2008).

9.2 *Eurydema ventrale* Kolenati

Cabbage stink bugs (*Eurydema ventrale* Kolenati) appear on different species of the family Brassicaceae. They cause damage primarily to young plants. Until recently, it was common practice to suppress stink bugs with chemical insecticides if they attacked massively (Maceljski *et al*., 2004).

Our study found out that EPNs can be efficient in suppression of stink bugs. Different factors influence the efficiency of nematodes. We found out that the efficiency of nematodes in suppression of stink bugs depends on the species and the concentration of nematode suspension, temperature, developmental stage of stink bugs and the time passed after the application. Nematodes were more efficient at higher temperatures (25 °C: 34 %) than at lower (20 °C: 13 %; 15 °C: 5 %). *Steinernema feltiae* at 15 °C and 20 °C showed the highest efficiency (57 %) in suppressing larvae of stink bugs. This cannot be confirmed for suppressing adult specimens of the harmful pest, since *Steinernema carpocapsae* was at 15 °C and 20 °C more successful (30 %). Nematodes were on average more efficient (23 %) at higher (2000 IJs/specimen) than at lower concentration (200 IJs/specimen; 13 %) (Zupančič, 2008).

9.3 Colorado potato beetle (*Leptinotarsa decemlineata* [Say])

The Colorado potato beetle (*Leptinotarsa decemlineata* [Say]) is in the majority of European countries still the most important harmful pest on potato (OEPP/EPPO, 1997). Larvae and adult specimens feed on foliage and thus retard development of plants. The first generation specimens are particularly harmful, so their economic threshold of harmfulness is less lower than the second generation specimens (Maceljski *et al*., 2004). The effect of EPNs on Colorado potato beetles has not been much researched in Europe. The results of our study (Trdan *et al*., 2009) show that both environmental temperature and the developmental stage of Colorado potato beetles importantly influence the efficiency of EPNs in suppression of this harmful pest. The lowest temperature, 15 °C, in this regard proved least appropriate, while at the temperatures 20 and 25 °C the efficiency EPNs was highest, which coincides with the results of our previous studies (Trdan *et al*., 2006), as well with the results of studies by other authors (Kaya and Gaugler, 1993). If we want to suppress the first (hibernated) adult specimens of the Colorado potato beetle by foliar application of the studied biological

agents – this is lately becoming widely-spread in suppression of harmful insects (Broadbent and Olthof, 1995) – it is advisable to apply a suspension of *S. feltiae* at higher concentrations. This species in our study at 15°C displayed the highest efficiency in suppression of adult specimens. When the first adult specimens appeared (the second half of May), nights in the region where we conducted the experiment are relatively fresh. Young larvae were at the lowest temperature most sensitive to nematodes, and they were first to react to nematodes at all temperatures. Older larvae were in this regard slightly less sensitive, yet still more than adult specimens – entomopathogenic nematodes are known to be more efficient with larvae than adult specimens (Trdan *et al*., 2009) because they more easily penetrate the former than the latter (LeBeck *et al*., 1993).

After *Steinernema feltiae* became autochthonous in Slovenia, we carried out the first field experiment with their use in Slovenia – for suppressing the Colorado potato beetle *Leptinotarsa decemlineata* (Say) (Laznik *et al*., 2010c). In our previous laboratory study we confirmed that EPNs are efficient biological agent for suppressing the said harmful insects (Trdan *et al*., 2009). The results of our study have shown that the Slovenian strain *Steinernema feltiae* (B30) and the commercial preparation Entonem (*S. feltiae*) are efficient biological agents for suppressing above-ground insects, particularly larvae stages, of Colorado potato beetles outdoors. Though some previous laboratory studies confirmed efficiency of EPNs also with adult specimens of Colorado potato beetles (Stewart *et al*., 1998; Trdan *et al*., 2009), the results of laboratory experiments can be hardly compared with those which are obtained outdoors (Cantelo and Nickle, 1992).

The results of our study have shown that the activity of EPNs on younger larvae at high concentrations is similarly fast as with the insecticide thiamethoxam, while the activity of nematodes on older larvae was delayed. EPNs and the insecticide thiamethoxam in our experiment did not displayed apparent activity, which had been already established by many other researchers (Armer *et al*., 2004; Hoffmann *et al*., 2008). The concentration of nematode suspension did not influence the larvae's and adult specimens' mortality rates, which is favourable from the aspect of economical use of the said biological agents in integrated food production as the costs of suppression of harmful pests with EPNs are proportionate with the quantity of used EPNs. That is precisely why we during the second repetition decided to apply halved concentration of nematode suspension. The influence of concentration of nematodes suspension on the mortality rates of insects can be explained by the fact that only a few IJs suffice to cause the insect's death (Bednarek and Nowicki, 1986; Arthurs *et al*., 2004). Despite lower price of EPNs during our second application, the ratio between the price of thiamethoxam/ha in comparison with the price of EPNs /ha remained 1:142.

In connection with changes in the population of Colorado potato beetles in our experiment we studied also the influence of the harmful pest on the mass of yield. The yield was in the year 2008 (Figure 1) larger than in the year 2009 (Figure 2), primarily as the consequence of planting our own seed material (Milošević *et al*., 2008). In comparison with some related studies, which researched the mass of yield of the Kondor potato (Ábrahám *et al*., 2006), we achieved lesser results, which can be to a large extent attributed to great population pressure of Colorado potato beetles in our experiment. No differences in influence on total tuber yield were found between individual treatments with EPNs. The mass of yield was also not influenced by the concentration of nematode suspension, which coincides with the fact that the population dynamics of larvae and adult specimens in our experiment, which

influence defoliation of potato plants most (Hare, 1980), was not affected by the concentration of nematode suspension. It is known that total defoliation of potato plants can cause more than 50 % loss of yield (Cranshaw and Redcliff, 1980).

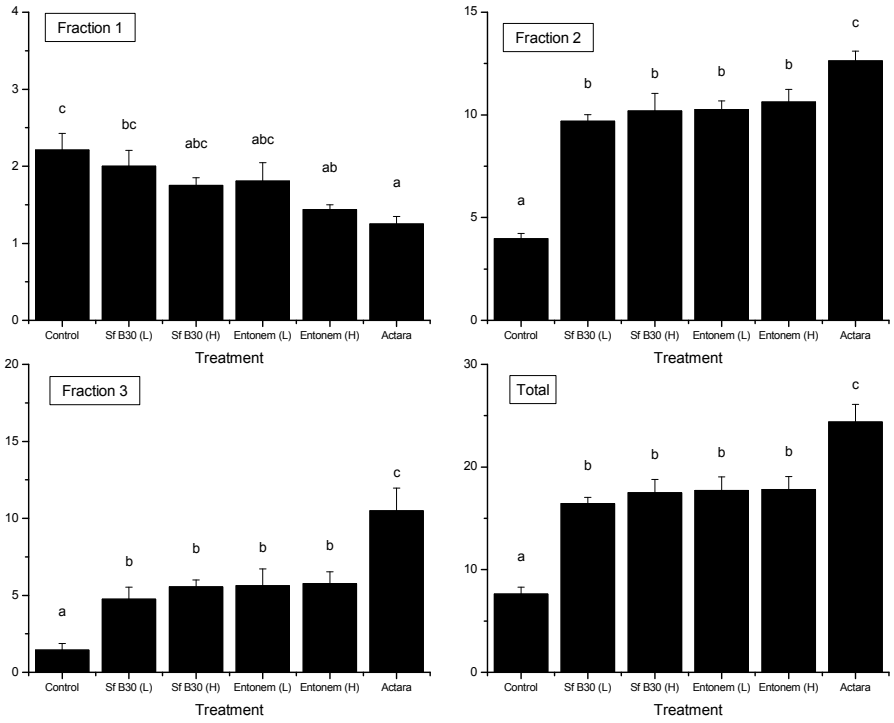


Fig. 1. Potato yield at different fractions and treatments in 2008 in t/ha (Fraction 1: size of tubers < 4 cm; Fraction 2: size of tubers between 4 and 5 cm; Fraction 3: size of tubers > 5 cm). The letters above the columns represent statistically significant differences between individual treatments.

The population of Colorado potato beetles in our experiment increased with time, and the majority of damage was done in the middle of potato's growth period (the end of May, June and the beginning of July) (Maceljski *et al.*, 2004), so we could reasonably expect loss of yield. We found out that more damaged plants (the control treatment) formed larger mass of smaller tubers, while the mass of larger tubers was smaller in the same treatment. The opposite was established in the treatments when plants were less damaged for a prolonged period of time and the formation of smaller tubers was not so intensive, while the mass of commercially more interesting (larger) tubers was higher. Total defoliation of potato influenced the development of tubers – sprouts formed ever anew, but they could not develop large tubers. The experiment confirmed the already known fact that the size of potato tubers is greatly influenced by the degree of success in suppressing larvae and adult specimens of Colorado potato beetles, since defoliation retards the development of tubers in soil (Mannan *et al.*, 1992). Total defoliation of potato plants occurred at the end of the

experiment in all treatments because the population of Colorado potato beetles on control blocks multiplied to the extent when it searched food (fresh potato leaves) in the immediate vicinity – this is consistent with the findings of related studies (Armer *et al*., 2004) in which the efficiency of *Heterorhabditis marelatus* Liu & Berry against Colorado potato beetles was researched. Despite the fact that Welch and Briand (1961) warn against foliar application of EPNs, since nematode suspension on leaves dries to quickly and is thus less efficient, we maintain that by taking into account key limiting factors (temperature, moisture, UV radiation) (Kaya and Gaugler, 1993) and by the appropriate way of application satisfactory results can be reached also with the foliar use of EPN. This was confirmed also by some other studies (Broadbent and Olthof, 1995; Arthurs *et al*., 2004). The greatest difficulty in using such biological preparations is still their lesser efficiency in comparison with chemical preparations and high price (Ehlers, 1998).

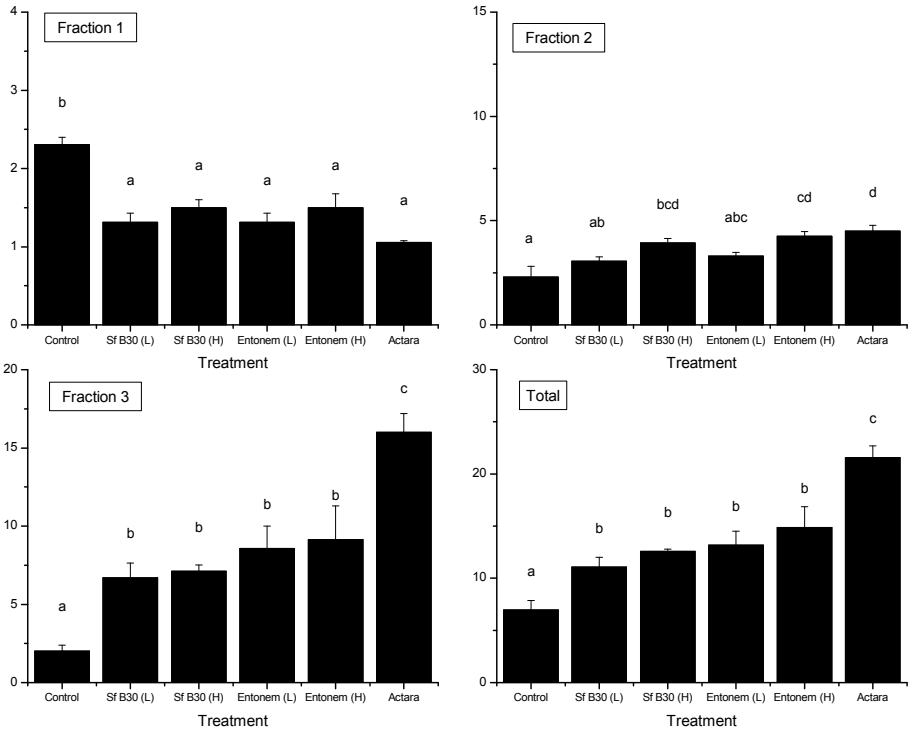


Fig. 2. Potato yield at different fractions and treatments in 2009 in t/ha (Fraction 1: size of tubers < 4 cm; Fraction 2: size of tubers between 4 and 5 cm; Fraction 3: size of tubers > 5 cm). The letters above the columns represent statistically significant differences between individual treatments.

9.4 Cereal leaf beetle (*Oulema melanopus* [L.])

The Cereal leaf beetle (*Oulema melanopus*) is widely spread in the world. In Europe it appears in all regions where cereals are grown, it is most widely spread in the Balkans and

the neighbouring regions, especially in those with continental and moderate continental climate (Olfert *et al*., 2004). Beetles and larvae usually feed on the same hosts. The principal damage is caused by larvae, which feed on intervenous tissue on upper sides of leaves. This can cause 9.5 % loss of yield. If 12 to 25 % of leaf surface of wheat is destroyed, the yield is reduced by 14 %. When the upper leaf if completely destroyed, which is the most frequently attacked organ of cereals (the density of the harmful pest decreases from the top of plants), the yield may be reduced by up to 60 % (Casagrande *et al*., 1977).

The results of our study, which researched effects of entomopathogenic nematodes in laboratory on adult specimens of the harmful pest (Laznik *et al*., 2010b), show that the mortality rates of the cereal leaf beetle imagos depends primarily on temperature, yet in connection with the concentration of nematode suspension, the nematode strain and the day after the treatment. All four studied strains caused the highest average mortality rates of specimens (81 %) six days after the treatment and at the highest concentration of nematode suspension (78 %). The most efficient among the studied strains was *S. carpocapsae* C101, which is caused 96 % mortality rate of beetles. The strain *H. bacteriophora* D54, on the other hand, caused only 49 % mortality rate in the studied insects. The comparison between the two strain of *S. feltiae* shows that the commercial preparation Entonem is more efficient than the Slovenian strain B30 (67 % and 54 %). At 15 and 20 °C we recorded lower mortality rate of beetles than at 25 °C, which coincides with the Slovenian studies so far (Trdan *et al*., 2008, 2009), while the strain *H. bacteriophora* D54 caused the highest mortality rate at higher temperatures. This too coincides with the results of the related studies (Trdan *et al*., 2008).

For imagos of cereal leaf beetles which hibernate and are in spring the first to appear on cereals, it is advisable to apply suspensions of nematodes *S. feltiae* and *S. carpocapsae*, since our experiment shows that these species reach highest efficiency at 15 °C. The first adult specimens appear in Middle and South Europe in the first half of April, when nights are still relatively fresh (Stamenković, 2004). In our experiment the high concentration of nematode suspension proved to be most efficient, though relatively satisfactory efficiency was observed also at lower concentrations (from 53 to 65 %).

On the basis of our study's results we can conclude that efficiency of EPNs depends more on the temperature than on the concentration of nematode suspension, but it seems that the influence of concentration is species-specific (Arthurs *et al*., 2004). The data show that some species of nematodes functioned equally well at both lower and higher concentration. This is from the aspect of economic use of entomopathogenic nematodes particularly important. We should not, however, neglect the important fact that laboratory results are not always comparable with field experiments (Cantelo and Nickle, 1992), since the efficiency EPNs outdoors depends on many other factors and interaction between them. In a similar experiment *S. carpocapsae* was 100 % efficient in suppressing larvae, pupae and adult specimens of Colorado potato beetles, while it reached only 31 % efficiency when its activity was tested outdoors (Stewart *et al*., 1998).

9.5 Common cockchafer (*Melolontha melolontha* [L.])

In Slovenia, common cockchafer (*Melolontha melolontha* [L.]) is one of the most economically important pest of grasslands. Control of common cockchafer is feasible with the application of insecticide. However, due to the appearance of insect resistance, efficacy decrease owing to soil microorganisms activity and doubts on environmentally acceptability of such kind products, alternative solution are sought for its control (Koppenhöfer and Kaya, 1998).

Results of our laboratory research (Laznik *et al*., 2009d) demonstrated that indigenous strain *S. feltiae* C76 attained higher mortality rate (27 %) of third-stage larvae of common cockchafer than commercial product Entonem (20 %). In a similar research, Berner and Schnetter (2001) reported on 3 % larval mortality when *S. feltiae* strain Ehlers was applied and that as the best nematode in their experiment proved to be *S. glaseri* strain RS92 (60 %). Reason for poorer activity of *S. feltiae* we can attribute to the fact, that it goes for the species which has not been found in naturaly infected white grubs as this is documented for *S. anomali* (Kozodoi) *S. glaseri* (Steiner), *S. kushidai* (Mamiya), *S. scarabaei* (Stock), and *Heterorhabditis megidis* (Poinar) (Poinar, 1975).
Differences between strains studied in our experiment can be found due to the fact that strain C76 is much bettter adapted to the larvae of common cockchafer as we confirmed its finding in the area (Laznik *et al*., 2009d), where in the past common cockchafer caused quite an extensive damage on grasslands (Urek and Milevoj, 1993). Grewal *et al*. (2004) came to similar conclusions, namely that different strains of the same EPN species might act differently on various insect pests. It was established multiple times that indigenous strains are more virulent from the exotic strains (Grewal *et al*., 2004).
In our experiment, the most promising activity demonstrated the strain C67 at 20 °C and at highest concentration of nematode suspension (53 %), meanwhile the highest effect of bioproduct Entonem was attained at 20 °C and at middle concentration of nematode suspension (31 %). At corresponding application *S. feltiae* can very satisfying control the younger larval stages of common cockchafer, but when compared to entomopathogenic fungus *Beuveria brongniartii* (Poženel, 2007), the efficacy of the nematodes is lower.

10. Activity of entomopathogenic nematodes against stored products pests

10.1 Rice weevil (*Sitophilus oryzae* [L.])

Rice weevil (*Sitophilus oryzae* [L.]) in the recent years represents one of the most important stored cereals pests in Europe (Stejskal *et al*., 2003). Some previous studies have shown that EPNs can be efficient biological agents for suppressing some species of stored products pests, such as *Sitophilus granarius* (L.) (Trdan *et al*., 2006), *Tribolium confusum* Jacquelin du Val (Athanassiou *et al*., 2007), *Tenebrio molitor* L., *Tribolium castaneum* (Herbst), *Trogoderma variabile* Ballion and *Oryzaephilus surinamensis* (L.) (Ramos-Rodriguez *et al*., 2006; Trdan *et al*., 2006). The results of our laboratory study, however, have shown that the mortality rates of adult specimen of rice weevil depend on temperature and concentrations of nematode suspension, as well as on the strain of nematodes (Laznik *et al*., 2010a). Similar findings were produced also by researchers in some related studies (Arthurs *et al*., 2004; Laznik *et al*., 2010b).
The lowest mortality rate was in our experiment noted at 30 °C, at which temperature no strain exceeded 20 % efficiency. Lower efficiency at high temperature can be attributed to the fact that the temperature interval in which EPN's activity is optimal ranges between 20 and 26 °C (Trdan *et al*., 2008; Laznik *et al*., 2010b), it is nonetheless a species-specific relation (Kaya and Gaugler, 1993). At the lowest temperature in the experiment (15 °C) the Slovenian strains of *S. feltiae* (B30 and B49) proved more efficient than the Hungarian strain 3162. The reason for lower efficiency lies also in the fact that the strain 3162 was isolated in Hungary, which has continental climate with higher average temperatures than Slovenia (Peel *et al*., 2007). This strain has during its evolution most probably adjusted to slightly higher temperatures. Similar conclusions were made also by Hazir *et al*. (2004), who found out that

strains of EPNs isolated in warmer regions function more efficiently at higher temperatures that those isolated in colder regions.

10.2 Granary weevil (*Sitophilus granarius* [L.]) and sawtoothed grain beetle (*Oryzaephilus surinamensis* [L.])

The granary weevil, *Sitophilus granarius* (L.), and the sawtoothed grain beetle, *Oryzaephilus surinamensis* (L.), are listed among important pests of stored grain, the former being a primary pest (Ungsunantwiwat and Mills, 1985) spread mostly in moderate climate, and the latter being a typical cosmopolitan and a secondary pest (Trematerra *et al.,* 2000). Both species feed on a variety of cereals or cereal products, although wheat and barley are among the most frequent sources of their nutrition (Schwartz and Burkholder, 1991).

The investigation demonstrated that entomopathogenic nematodes control the granary weevil adults more efficiently than they control the adults of the sawtoothed grain beetle. The application of *S. feltiae*, *S. carpocapsae* and *H. bacteriophora* at 20 °C and 25 °C resulted in mortality rates of over 57 % in the granary weevil beetles. Satisfactory results in the control of the sawtoothed grain beetle were achieved only at 20 °C, with the percentage of mortality of the beetles ranging between 44 and 81 %. These outcomes partly agree with the results of some related works, where an optimal biological activity of *S. carpocapsae, H. bacteriophora,* and *S. feltiae* was determined in the temperature range from 22 to 24°C (Choo HoYul et al. 2002), from 22 to 26 °C (Kaya and Gaugler, 1993), and at 25°C (Belair *et al*., 2003), respectively. In higher concentrations (500 IJs/adult and more) entomopathogenic nematodes can be considered efficient biological agents to control the adults of *S. granarius* and *O. surinamensis*. The general impression is that the use of the highest nematode concentrations (2000 IJs/adult) is not economically justified. *S. feltiae*, *S. carpocapsae* and *H. bacteriophora* can be recommended for the storage pest control since they were the most effective in the control of both storage pests.

11. Non-target effect of entomopathogenic nematodes

Entomopathogenic nematodes have been proven effective in controlling some foliar pests (Trdan *et al.,* 2007a; Laznik *et al*., 2010c; Laznik *et al.,* 2011), but they do have some negative properties. Among these, the wide spectrum of their efficacy includes a negative influence on beneficial organisms (Hazir *et al*., 2004). Up to now, the studies on the non-target effects of entomopathogenic nematodes were performed on various species of non-target organisms, and a large range - from complete harmlessness to pronounced harmful effect - was established (Bathon 1996; Farag 2002). The results of some field trials show a moderate influence of entomopathogenic nematodes on non-target arthropods or even the absence of such an effect (Georgis *et al.* 1991). Bathon (1996) reports that mortality can be observed among the non-target organisms, but the influence of these agents should be temporary and local and so only a part of the population is under attack. Georgis *et al.* (1991) demonstrated a negligible influence of entomopathogenic nematodes on non-target organisms if they are used only in short term pest control.

Farag (2002) reports a high mortality of the larvae of *Coccinella undecimpunctata* Linnaeus caused by *Heterorhabditis taysearae* Shamseldean and *Steinernema carpocapsae* strain S2 in a laboratory assay, so the author does not recommend the use of entomopathogenic nematodes when these predators are present on the plants in high number. Likewise, *Heterorhabditis bacteriophora* Poinar and *Steinernema carpocapsae* (Weiser) species were – under

laboratory conditions – very harmful to the following predators: *Coleomegilla maculata* [De Geer], *Olla v-nigrum* [Mulsant], *Harmonia axyridis* [Pallas] and *Coccinella septempunctata* L. On the other side Shapiro-Ilan and Cottrell (2005) found the lady beetles to be substantially less susceptible to nematode infection compared with a known susceptible insect - the black cutworm (*Agrotis ipsilon* Hüfnagel).
Results of our investigation showed that larvae of the two-spotted lady beetle and green lacewing are susceptible to EPN attack under laboratory conditions. The mortality rate for the two-spotted lady beetle larvae at 25°C was over 93%. The mortality rate for the green lacewing larvae at the both higher temperature was over 42%. In the most cases *H. bacteriophora* and mixed suspension of *S. feltiae* and *H. bacteriophora* were the least non-targetly efficient agents in our research (Rojht *et al.,* 2009).

12. Possibilities for integrated plant protection with entomopathogenic nematodes in future?

Entomopathogenic nematodes may be combined with other agricultural chemicals and control agents for variour purposes. Based on our research, we conclude that the benefits of utilizing EPNs combined fungicide can offer better control management of an IPM programme. EPNs can be applied with nearly all commercially available ground or aerial spray equipment, including pressurized sprayers, mist blowers, and electrostatic sprayers (Kaya and Gaugler, 1993). This offers a cost-effective alternative to pest control, while simultaneous application of fungicide and EPNs influence as well on fungi agents of plant diseases as on insects, and it saves time and at the same time money spent for controlling pest organisms (Kaya and Gaugler, 1993). For active ingredients azoxystrobin (Anand *et al*., 2008), propamocarb (Urban and Lebeda, 2007), and sulphur (Bassino *et al*., 1977) are well known their efficacy against cucumber downy mildew (*Pseudoperonospora cubensis* [(Berk. & M.A. Curtis) Rostovzev]) and powdery mildew disease (*Erysiphe cichoracearum* DC). While several precedent research showed that EPNs when applied correctly act efficiently on some pests of cucumbers, for example western flower thrips (*Frankliniella occidentalis* Pergande) (Trdan *et al*., 2007), and on the greenhouse whitefly (*Trialeurodes vaporariorum* [Westwood]) (Laznik *et al*., 2011). Synchronous application of fungicide and EPNs in such examples for controlling the aforementioned pests is justifiable. Similar conclusions can also be drawn together when dealing with active ingredients metiram, copper hydroxide, and mancozeb, which control especially potato blight (*Phytophthora infestans* [Mont.] de Bary), lettuce downy mildew (*Bremia lactucae* Regel), wheat leaf blotch (*Septoria tritici* Thüm.), and early blight of tomatoes (*Alternaria solani* Sorauer) (Milus, 1994; Stepanović *et al*., 2009; Stevenson, 2009) in connection with some pest insects like the Colorado potato beetle (*Leptinotarsa decemlineata* [Say]), cabbage armyworm (*Mamestra brassicae* [L.]), barley wireworm (*Agriotes fuscicollis* Miwa), cereal leaf beetle (*Oulema melanopus* [L.]), the tomato leafminer (*Tuta absoluta* [Povolny]) for which some previous research showed that EPNs are very effective biological agents for their control (Batalla-Carrera *et al*., 2010; Laznik *et al*., 2010ab). Anyway, future implementation of synchronous application of EPNs and fungicides must be supported by field experiments, while results of laboratory experiments can not be transferred uncritically into conditions which hold for the environment.
It is well known that biological preparations at the moment represent only 1% of the world market for protection of plants against diseases, harmful pests and weeds (Dent, 2003). As much as 80 % of all biological preparations are based on the active substance of the bacteria

Bacillus thuringiensis (*Bt*). Many analysts are of the opinion that biological preparations could replace as much as 20 % of chemical products available on the market which is worth 7 billion American dollars (Blum, 2002). The dismal fact is that industrial plants which produce biological preparations are still insufficiently equipped and not prepared to penetrate such a big and important market. Even greater problem is the lack of awareness on the part of food producers and consumers regarding the significance of biological control (Dent, 2003). The use of nature-friendly organisms in plant protection is at the moment limited, this is primarily due to the fact that general public is not familiar with the usefulness of entomopathogenic nematodes as a means of biological protection of plants, as well as to the prevailing opinion that they are not as efficient as chemical products.

13. Conclusion

The results of many foreign studies and the Slovenian studies presented in this chapter prove that entomopathogenic nematodes are, when applied in optimal conditions, efficient agents for suppressing harmful pests (Kaya and Gaugler, 1993). Of course we must be aware that they are in general not as efficient as chemical insecticides, although some studies have also shown that appropriate applications of entomopathogenic nematodes can provide mortality rates of target harmful pests which are comparable with mortality rates of organisms exposed to synthetic insecticides (Laznik *et al.*, 2011). When deciding to use entomopathogenic nematodes or even implementing them into the systems of food production, our expectations should not be the same as when using conventional protection of plants. We particularly should not focus solely on greater yields, but also bear in mind that using the said agents is acceptable for food, man and nature. In this case we should be prepared to tolerate slightly less attractive appearance of plants grown for food or decoration. At present we no longer have insecticides for suppressing many harmful insects, this means that circumstances are exceptionally favourable to increase the use of natural enemies and other forms biological protection of plants, and entomopathogenic nematodes are in this regard one of the most efficient alternatives to insecticides. The results of foreign and Slovenian studies show that these agents deserve to be given a try!

14. Acknowledgement

This chapter was performed under the projects L4-6477 and V4-0524, funded by the Slovenian Research Agency and Ministry of Agriculture, Food, and Forestry of the Republic of Slovenia, project V4-1067, funded by the Slovenian Research Agency, Ministry of Agriculture, Food, and Forestry of the Republic of Slovenia and Ministry of the Environment and Spatial Planning of Republic of Slovenia, and the programme Horticulture (P4-0013) funded by the Slovenian Research Agency. Part of the researches presented in this chapter was funded within Professional Tasks from the Field of Plant Protection, a program funded by the Ministry of Agriculture, Forestry, and Food of Phytosanitary Administration of the Republic of Slovenia, and within bilateral co-operation in science and technology between Slovenia and Hungary in the period 2008-2011. We thank Dr. Matej Vidrih, Dr. Filip Vučajnk, Dr. Dragan Žnidarčič, Dr. Tamas Lakatos, Dr. Timea Tóth, Jaka Rupnik, Aleksander Bobnar, Helena Rojht MSc, Melita Štrukelj, Sanja Ljubi, Luka Kužnik, Alenka Zupančič, Petra Premrl, and Nevenka Valič for technical assistance.

15. References

Abbott, W.S. 1925: A method of computing the effectiveness of an insecticide. Journal of Economic Entomology, 18: 265-267.

Ábrahám E.B., Zsom E., Sárvári M. 2006. The effect of year and irrigation on the yield quantity and quality of the potato. Journal of Agricultural Sciences, 24: 12-16

Akhurst R. J. 1980. Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. Journal of General Microbiology, 121: 303-309

Anand T., Chandrasekaran A., Pillai-Kuttalam S., Senthilraja G., Raguchander T., Samiyappan R. 2008. Effectiveness of azoxystrobin in the control of *Erysiphe cichoracearum* and *Psedoperonospora cubensis* on cucumber. Journal of Plant Protection Research, 48: 147-158.

Armer C.A., Berry R.E., Reed G.L., Jepsen S.J. 2004. Colorado potato beetle control by application of the entomopathogenic nematode *Heterorhabditis marelata* and potato plant alkaloid manipulation. Entomologia Experimentalis et Applicata, 111: 47-58

Arthurs S., Heinz K.M., Prasifka J.R. 2004. An analysis of using entomopathogenic nematodes against above-ground pests. Bulletin of Entomological Research, 94: 297-306

Athanassiou C.G., Palyvos N.E., Kakouli-Duarte T. 2007. Insecticidal effect of *Steinernema feltiae* (Filipjev) (Nematoda: Steinernematidae) against *Tribolium confusum* du Val (Coleoptera: Tenebrionidae) and *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) in stored wheat. Journal of Stored Product Research, 44: 52-57

Athanassiou, C.G., Kavallieratos, N.G., Menti, H., Karanastasi E. 2010. Mortality of four stored product pests in stored wheat when exposed to doses of three entomopathogenic nematodes. Journal of Economic Entomology 103: 977-984.

Bassino J.-P., Bailly R., Blanc M., Pascal C. 1977. Powdery mildew of melon. The efficiency of various products. Defense des Vegetaux 31: 151-157.

Batalla-Carrera L., Morton A., Garcia del Pino F. 2010. Efficacy of entomopathogenic nematodes against the tomato leafminer *Tuta absoluta* in laboratory and greenhouse conditions. BioControl 55: 523-530.

Bathon H. 1996. Impact of entomopathogenic nematodes on non-target hosts. Biocontrol Science and Technology, 6: 421-434

Bedding R.A., Akhurst R.J. 1975. A simple technique for the detection of insect parasitic rhabditid nematodes in soil. Nematologica, 21: 109-110

Bednarek A., Nowicki T. 1986. Effect of intrapopulation factors in the nematodes *Steinernema feltiae* (Steinernematidae) on the intensity of insect infestation. Zeszyty Problemowe Postepow Nauk Rolniczych, 323: 199-212

Belair, G., Fournier, Y., Dauphinais, N. (2003): Efficacy of steinernematid nematodes against three insect pests of crucifers in Quebec. Journal of Nematology, 35, 259-265.

Berner, M., Schnetter, W. 2001. Wirksamkeit entomopathogener Nematodes gegen Engerlinge der Maikäfer *Melolontha melolontha* und *M. hippocastani*. Mitt. Dtsch. Ges. Allg. angew. Entomol. 13: 165-167.

Blum, B. 2002. Blocked opportunities for bio-control. Pesticide News 57, September 2002.

Boemare, N.E., Akhurst, R.J., Mourant, R.G. 1993. DNA relatedness between *Xenorhabdus* spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, and

a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. Nov. International Journal of Systematic Bacteriology, 43: 249-255.

Boemare N.E., Laumond C., Luciani J. 1982. Mise en evidence d'une toxicogenese provoquee par le nematode entomophage *Neoplectana carpocapsae* Weiser chez l'insecte *Galleria mellonella* L. Compets Rendus des séances de l'Academie des Scences, Paris, Ser. III. 295: 543-546

Bovien, P. 1937. Some type of association between nematodes and insects. Videnk. Meddr Naturh. Foren. 101: 1-114.

Broadbent A. B., Olthof T. H. A. 1995. Foliar application of *Steinernema carpocapsae* (Rhabditida: Steinernematidae) to control *Liriomyza trifolii* (Diptera: Agromyzidae) larvae in chrysanthemums. Environmental Entomology, 24: 431-435

Campbell J. F., Lewis E. E., Stock S. P., Nadler S., Kaya H. K. 2003. Evolution of host search strategies in entomopathogenic nematodes (Nematoda: Steinernematidae). Journal of Nematology, 35: 142-145

Cantelo W.W., Nickle W.R. 1992. Susceptibility of prepupae of the Colorado potato beetle (Coleoptera: Chrysomelidae) to entomopathogenic nematodes (Rhabditida: Steinernematidae, Heterorhabditidae). Journal of Entomological Science, 27: 37-43

Cantliffe, D.J., Funes, J., Jovicich, E., Paranjpe, A., Rodriguez, J., Shaw, N. 2003. Media and containers for greenhouse soilless grown cucumbers, melons, peppers and strawberries. Acta Horticulturae 614: 199-203.

Casagrande R.A., Ruesink W.G., Haynes D.L. 1977. The behaviour and survival of adult cereal leaf beetles. Annals of the Entomological Society of America, 70: 19-30

Chavarría-Hernandez, N., M. de la Torre, 2001: Population growth kinetics of the nematode, *Steinernema feltiae*, in submerged monoxenic culture. Biotechnol. Lett. 23, 311-315.

Choo HoYul, Lee DongWoon, Yoon HeeSook, Lee SangMyeong, Hang DaoThi, 2002. Effects of temperature and nematode concentration on pathogenicity and reproduction of entomopathogenic nematode, *Steinernema carpocapsae* Pocheon strain (Nematoda: Steinernematidae). Korean Journal of Applied Entomology, 41, 269-277.

Chyzik, R., Glazer, J., Klein, M. 1996. Virulence and efficacy of different entomopathogenic nematode species against western flower thrips (*Frankliniella occidentalis*). Phytoparasitica 24: 103-110.

Coffin, R.S., Coutts, R.H.A. 1995. Relationships among *Trialeurodes vaporariorum*-transmitted yellowing viruses from Europe to North America. J. Phytopathol. 143: 375-380.

Cranshaw W.S., Radcliffe E.B. 1980: Effect of defoliation on yield of potatoes. Journal of Economic Entomology, 73: 131–134

De Nardo, E.A.B., Grewal, P.S., 2003. Compatibility of *Steinernema feltiae* (Nematoda: Steinernematidae) with Pesticides and Plant Growth Regulators Used in Glasshouse Plant Production. Biocontrol Sci. Technol. 13, 441-448.

Dent, D. 2003. Can biological control replace chemicals? Edited presentation to the Pesticide Challenge conference.

Ehlers R.-U. 2001. Mass production of entomopathogenic nematodes for plant protection. Applied Microbiology and Biotechnology, 56: 523-633

Fan, X., Hominick, W.M. 1991. Efficiency of the *Galleria* (wax moth) baiting technique for recovering infective stages of entomopathogenic rhabditids (Steinernematidae and Heterorhabditidae) from sand and soil. Rev. Nematol., 14: 381-387.

Farag N. A. 2002. Impact of two entomopathogenic nematodes on the ladybird, *Coccinella undecimpunctata* and its pray, *Aphis fabae*. Ann. Agric. Sci. (Cairo). Fac. Agric., Ain Shams Univ., Cairo, Egypt, 47: 431-443

Fischer-Le Saux M., Mauléon H., Constant P., Brunel B., Boemare N.E. 1998. PCR-ribotyping of *Xenorhabdus* and *Photorhabdus* isolates from the Caribbean region in relation to the taxonomy and geographic distribution of their nematode hosts. Applied Environmental Microbiology, 64: 4246-4254

Flanders, K.L., Miller, J.M., Shields, E.J. 1996. *In vivo* production of *Heterorhabditis bacteriophora* »Oswego« (Rhabditida: Heterorhabditidae), a potential biological control agent for soil-inhabiting insects in temperate regions. Journal of Economic Entomology, 89: 373-380.

Forst S., Clarke D., 2002. Bacteria-nematode symbiosis. In: Entomopathogenic Nematology. Gaulger R. (ed.). Wallingford, UK, CABI Publishing: 57-77

Georgis R., Kaya H. K., Gaugler R. 1991. Effect of steinernematid and heterorhabditid nematodes (Rhabditida: *Steinernematidae* and *Heterorhabditidae*) on nontarget arthropods. Environmental Entomology, 20: 815-822

Glaser, R.W., Farrell, C.C. 1935. Field experiments with the Japanese beetle and its nematode parasite. Journal of New York Entomological Society 43: 345.

Glaser R W., Fox H. 1930. A nematode parasite of the Japanese beetle (*Popillia japonica* Newm). Science, 71: 1617

Gorman, K., Hewitt, F., Denholm, I., Devine, G.J. 2001. New developments in insecticide resistance in the glasshouse whitefly (*Trialeurodes vaporariorum*) and the two-spotted spider mite (*Tetranychus urticae*) in the UK. Pest Management Science 58, 123-130.

Grewal, P.S., 2002. Formulation and application technology. In: Entomopathogenic Nematology CABI Publishing (Gaugler, R., Ed.) vol. 15. CAB International, Wallingford, 311-332.

Grewal, P.S., Power, K.T., Grewal, S.K., Suggars, A., Haupricht, S. 2004. Enhanced consistency in biological control of white grubs (Coleoptera: Scarabaeidae) with new strains of entomopathogenic nematodes. Biological Control 30: 73-82.

Grewal P.S., Ehlers R.-U., Shapiro-Ilan D.I. 2005. Nematodes as biocontrol agents. Wallingford, UK, CABI Publishing: 505 str.

Hare J.D. 1980. Impact of defoliation by the Colorado potato beetle on potato yields. Journal of Economic Entomology, 73: 369-373

Hara, A.H., Kaya, H.K., Gaugler, R., Lebeck, L.M., Mello, C.L. 1993. Entomopathogenic nematodes for biological control of leafminer, *Liriomyza trifolii* (Diptera: Agromyzidae). Entomophaga 38: 359-369.

Hazir S., Kaya H. K., Stock S.P., Keskin N. 2004. Entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) for biological control of soil pests. Turkish Journal of Biology, 27: 181-202

Hoffmann E.J., Middleton S.M., Wise J.C. 2008. Ovicidal activity of organophosphate, oxadiazine, neonicotinoid and insect growth regulator chemistries on northern strain plum curculio, *Conotrachelus nenuphar*. Journal of Insect Science, 8: 1536-2442

Hominick W.M. 2002. Biogeography. V: Entomopathogenic Nematology. Gaugler R (ed). Wallingford, UK, CABI Publishing:115-143

Hominick W.M., Briscoe B.R., del Pino F.G., Heng J., Hunt D.J., Kozodoy E., Mracek Z., Nguyen K.B., Reid A.P., Spiridonov S., Stock P., Sturhan D., Waturu C., Yoshida M. 1997. Biosystematics of entomopathogenic nematodes: current status, protocols and definitions. Journal of Helminthology, 71: 271-298.

Ishibashi N., Choi D.-R. 1991. Biological control of soil pests by mixed application of entomopathogenic and fungivorous nematodes. Journal of Nematology, 23: 175-181

Kaya H. K., Gaugler R. 1993. Entomopathogenic nematodes. Annual Review of Entomology, 38: 181-206

Koppenhöfer, A.M., Cowles, R.S., Cowles, E.A., Fuzy, E.M., Baumgartner, L., 2002. Comparison of neonicotinoid insecticides as synergists for entomopathogenic nematodes. Biol. Control 24, 90-97.

Koppenhöffer A.M., Fuzy E.M., Crocker R., Gelernter W., Polavarapu S. 2004. Pathogenicity of *Steinernema scarabaei*, *Heterorhabditis bacteriophora* and *S. glaseri* to twelve white grub species. Biocontrol Science and Technology, 14: 87-92

Koppenhöfer, A.M., Kaya, H.K. 1998. Synergism of imidacloprid and an entomopathogenic nematode: A novel approach to white grub (Coleoptera: Scarabaeidae) control in turfgrass. Journal of Economic Entomology, 91: 618-623.

Laznik Ž., Trdan S. 2008a. Entomopathogenic and entomoparasitic nematodes as biological control agents of thrips. Acta Phytopathologica et Entomologica Hungarica, 43: 317-322

Laznik Ž., Trdan S. 2008b. Entomopathogenic nematodes, natural enemies of foliar pests of vegetable brassicas. Acta agriculturae Slovenica, 91: 227-237 [Slovenian]

Laznik Ž., Tóth T., Lakatos T., Trdan S. 2008. Entomopathogenic nematode *Steinernema carpocapsae* (Weiser) (Rhabditida: Steinernematidae), a new member of Slovenian fauna. Acta agriculturae Slovenica, 91: 351-359

Laznik Ž., Tóth T., Lakatos T., Vidrih M., Trdan S. 2009a. First record of *Steinernema feltiae* (Filipjev) (Rhabditida: Steinernematidae) in Slovenia. Helminthologia, 46:135-138

Laznik Ž., Tóth T., Lakatos T., Vidrih M., Trdan S. 2009b. First record of a cold active entomopathogenic nematode *Steinernema kraussei* (Steiner) (Rhabditida: Steinernematidae) in Slovenia. Acta agriculturae Slovenica, 93: 37-42

Laznik Ž., Tóth T., Lakatos T., Trdan S. 2009c. *Heterorhabditis bacteriophora* (Poinar) – the first member from Heterorhabditidae family in Slovenia. Acta agriculturae Slovenica, 93: 181-187

Laznik Ž., Tóth T., Lakatos T., Vidrih M., Trdan S. 2009d. Efficacy of two strains of *Steinernema feltiae* (Filipjev) (Rhabditida: Steinernematidae) against third-stage larvae of common cockchafer (*Melolontha melolontha* [L.], Coleoptera, Scarabaeidae) under laboratory conditions. Acta Agriculturae Slovenica, 93: 293-299

Laznik Ž., Tóth T., Lakatos T., Vidrih M., Trdan S. 2010a. The activity of three new strains of *Steinernema feltiae* against adults of *Sitophilus oryzae* under laboratory conditions. Journal of Food, Agriculture and Environment, 8: 132-136

Laznik Ž., Tóth T., Lakatos T., Vidrih M., Trdan S. 2010b. *Oulema melanopus* (L.) (Coleoptera: Chrysomelidae) adults are susceptible to entomopathogenic nematodes (Rhabditida) attack: results from a laboratory study. Journal of Plant Diseases and Protection, 117: 30-32

Laznik Ž., Tóth T., Lakatos T., Vidrih M., Trdan S. 2010c. Control of the Colorado potato beetle (*Leptinotarsa decemlineata* [Say]) on potato under field conditions: a

comparison of the efficacy of foliar application of two strains of *Steinernema feltiae* (Filipjev) and spraying with thiametoxam. Journal of Plant Diseases and Protection, 117: 129-135

Laznik Ž., Žnidarčič D., Trdan S. 2011. Control of *Trialeurodes vaporariorum* (Westwood) adults on glasshouse-grown cucumbers in four different growth substrates: an efficacy comparison of foliar application of *Steinernema feltiae* (Filipjev) and spraying with thiamethoxam. Turkish Journal of Agriculture and Forestry, 35 (in press)

LeBeck L. M., Gaugler R., Kaya H. K., Hara A. H., Johnson M. W. 1993. Host stage suitability of the leafminer *Liriomyza trifolii* (Diptera: Agromyzidae) to the entomopathogenic nematode *Steinernema carpocapsae* (Rhabditida: Steinernematidae). Journal of Invertebrate Pathology, 62: 58-63

Long S.J., Richardson P.N., Fenlon J.S. 2000. Influence of temperature on the infectivity of entomopathogenic nematodes (*Steinernema* and *Heterorhabditis* spp.) to larvae and pupae of the vine weevil *Otiorhynchus sulcatus* (Coleoptera: Curculionidae). Nematology, 2: 309-317

Maceljski, M., Cvjetković, B., Ostojić, Z., Igrc-Barčić, J., Pagliarini, N., Oštrec, L., Barić, K., Čizmić, I. 2004. Štetočinje povrća s opsežnim prakazom zaštite povrća od štetnika, uzročnika bolesti i korova. Čakovec, Zrinski: 517 pp.

Mannan M.A., Bhuiyan M.K.R., Quasem A., Rashid M.M., Siddique M.A., 1992. Study on the growth and partitioning of dry matter in sween potato. Journal of Root Crops, 18: 1-5

Mráček, Z., Bečvář, S. 2000. Insect aggregations and entomopathogenic nematode occurence. Nematology, 2: 297-301.

Milevoj L. 1998. Perspektive biotičnega varstva rastlin v Sloveniji. In: Zbornik posveta Kmetijstvo in okolje. Rečnik M. (ur.) in Verbič J. (eds.). Ljubljana, Kmetijski inštitut Slovenije: 163-171

Milevoj L. 2002. Biotično varstvo solatnic. Sodobno kmetijstvo, 33: 282-283

Milošević D., Djalović I., Bugarčić Z. 2008. The importance of a healthy planting material for potato production increases. Agro-knowledge Journal, 9: 5-17

Milus E.A., 1994. Effect of foliar fungicides on disease control, yield and test weight of soft red winter wheat. Crop Protection, 13: 291-295.

Nielsen O., Philipsen H. 2004. Occurrence of *Steinernema* species in cabbage fields and the effect of inoculated *S. feltiae* on *Delia radicum* and its parasitoids. Agricultural and Forest Entomology, 6: 25-30

O'Halloran D.M., Burnell A.M. 2002. Olfaction and odour discrimination in the insect parasitic nematode *Heterorhabditis bacteriophora*. Nematology, 4: 206

Olfert O., Weiss R.M., Woods S., Philip H., Dosdall L. 2004. Potential distribution and relative abundance of an invasive cereal crop pest, *Oulema melanopus* L. (Coleoptera: Chrysomelidae), in Canada. The Canadian Entomologist, 136: 277-287

Peel M.C., Finlayson B.L., McMahon T.A. 2007. Updated world map of the Köppen-Geiger climate classification. Hydrology and Earth System Sciences, 11: 1633-1644

Perme S. 2005. Testing the efficacy of entomopathogenic nematodes (Rhabditida) against foliar pests of vegetables. Master Thesis, University of Ljubljana, Biotechnical faculty: 89 pp.

Poinar, G.O. Jr. 1975. Entomogenous Nematodes. E.J. Brill, Leiden, The Netherlands: 317 pp.

Poinar G.O. 1988. Redescription of *Neoaplectana affinis* Bovien (Rhabditida: Steinernematidae). Revue de Nematology, 11: 143-147

Poinar, G.O. Jr. 1993. Origins and phylogenetic relationships of the entomophilic rhabditids, *Heterorhabditis* and *Steinernema*. Fundamental and Applied Nematology, 16: 333-338.

Poženel, A. 2007. Experiences in controlling common cockchafer (*Melolontha melolontha* L.) in Idrija region. Lect. pap. present. 8th Slov. Conf. Plant Prot., Radenci, March 6-7 2007. Ljubl., Plant Prot. Soc. Slov.: 72-77 [Slovenian]

Pravilnik o biotičnem varstvu rastlin. 2006. Ur. l. RS št. 45/06

Premachandra, W. T. S. D., Borgemeister, C., Berndt, O., Ehlers, R.-U., Poehling, H.-M., 2003. Laboratory bioassays of virulence of entomopathogenic nematodes against soil-inhabiting *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae). Nematology 5: 539-547.

Ramos-Rodriguez O., Campbell J.F., Ramaswamy S.B. 2006. Pathogenicity of three species of entomopathogenic nematodes to some major stored-product insect pests. Journal of Stored Product Research, 42: 241-252

Rojht, H., Kač, M. & Trdan, S. 2009: Nontarget effect of entomopathogenic nematodes on larvae of twospotted lady beetle (Coleoptera: Coccinellidae) and green lacewing (Neuroptera: Chrysopidae) under laboratory conditions. Journal of Economic Entomology, 102 (4): 1440-1443.

Rovesti, L., Deseo, K.K., 1990. Compatibility of chemical pesticides with entomopathogenic nematodes *Steinernema carpocapsae* Weiser and *S. feltiae* Filipjev (Nematoda: Steinernematidae). Nematologica 36: 237-245.

Schwartz B.E., Burkholder, W.E. (1991): Development of the granary weevil (Coleoptera: Curculionidae) on barley, corn, oats, rice, and wheat. Journal of Economic Entomology, 84, 1047-1052.

Shapiro-Ilan D.I., Cottrell T. E. 2005. Susceptibility of lady beetles (Coleoptera: Coccinellidae) to entomopathogenic nematodes. Journal of Invertebrate Pathology, 89: 150-156

Sher, R.B., Parella, M.P. 1999. Biological control of the leafminer, *Liriomyza trifolii*, in chrysanthemums: implications for intraguild predation between *Diglyphus begini* and *Steinernema carpocapsae*. Bulletin of the International Organization for Biological and Integrated Control of Noxious Animals and Plants: Integrated Control in Glasshouses, 22: 221-224.

Sklep o spremembi statusa tujerodnega organizma za biotično varstvo rastlin: št. 3430-9/2008/5. 2008a. Ljubljana, Ministrstvo za kmetijstvo; gozdarstvo in prehrano: 2 pp. (internal material)

Sklep o spremembi statusa tujerodnega organizma za biotično varstvo rastlin: št. 3430-583/2008/2. 2008b. Ljubljana, Ministrstvo za kmetijstvo; gozdarstvo in prehrano: 2 pp. (internal material)

Sklep o spremembi statusa tujerodnega organizma za biotično varstvo rastlin: št. 3430-2/2009/2. 2009a. Ljubljana, Ministrstvo za kmetijstvo; gozdarstvo in prehrano: 2 pp. (internal material)

Sklep o spremembi statusa tujerodnega organizma za biotično varstvo rastlin: št. 3430-12/2009/2. 2009b. Ljubljana, Ministrstvo za kmetijstvo; gozdarstvo in prehrano: 2 pp. (internal material)

Stamenković S. 2004. Occurrence and damage of cereal leaf beetle (*Oulema melanopus* L.). Biljni lekar, 32: 124-127

Statistični urad Republike Slovenije 2005. http://www.stat.si (28.1.2011)

Stejskal V., Hubert J., Kučerová Z., Munzbergová Z., Lukaš J., Žd'árková E. 2003. The influence of the type of storage on pest infestion of stored grain in the Czech Republic. Plant Soil and Environment, 49: 55-62

Stepanović M., Vuksa P., Potočnik I., Milijašević S., Rekanović E., Todorović B. 2009. Integrated management of viral sensitivity of *Alternaria solani* isolatess to some fungicides. Acta Horticulturae 808: 377-380.

Stevenson W.R. 2009. Late blight control strategies in the United States. Acta Horticulturae 834: 83-86.

Stewart J.G., Boiteau G., Kimpinski J. 1998. Management of late-season adults of the Colorado potato beetle (Coleoptera: Chrysomelidae) with entomopathogenic nematodes. The Canadian Entomologist, 130: 509-514

Sturhan D. 1996. Seasonal occurrence, horizontal and vertical dispersal of entomopathogenic nematodes in a field. Mitteilungen aus der Biologischen Bundesanstalt fur Land- und Forstwirtschaft, 317: 35-45

Tokumaru, S., Abe, Y. 2005. Effects of host plants on the development and host preference of *Liriomyza sativae*, *L. trifolii* and *L. bryoniae* (Diptera: Agromyzidae). Japanese Journal of Applied Entomology and Zoology 49: 135-142.

Trdan S., Vidrih M., Valič N. 2006. Activity of four entomopathogenic nematode species against young adults of *Sitophilus granarius* (Coleoptera: Curculionidae) and *Oryzaephilus surinamensis* (Coleoptera: Silvanidae) under laboratory conditions. Journal of Plant Diseases and Protection, 113: 168-173

Trdan S., Žnidarčič D., Vidrih M. 2007a. Control of *Frankliniella occidentalis* on glasshouse-grown cucumbers: an efficacy comparison of foliar application of *Steinernema feltiae* and spraying with abamectin. Russian Journal of Nematology, 15: 25-34

Trdan, S., Kužnik, L. & Vidrih, M. 2007b: First results concerning the efficacy of entomopathogenic nematodes against *Hercinothrips femoralis* (Reuter). Acta agriculturae Slovenica, 89: 5-13.

Trdan S., Vidrih M., Valič N., Laznik Ž. 2008. Impact of entomopathogenic nematodes on adults of *Phyllotreta* spp. (Coleoptera: Chrysomelidae) under laboratory conditions. Acta Agriculturae Scandinavica, B Soil Plant Science, 58: 169-175

Trdan S., Vidrih M., Andjus L., Laznik Ž. 2009. Activity of four entomopathogenic nematode species against different developmental stages of Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera, Chrysomelidae). Helminthologia, 46: 14-20

Trematerra, P., Sciarreta, A., Tamasi, E. (2000): Behavioural responses of *Oryzaephilus surinamensis*, *Tribolium castaneum* and *Tribolium confusum* to naturally and artificially damaged durum wheat kernels. Entomologia Experimentalis et Applicata 94: 195-200.

Ungsunantwiwat, A., Mills, R.B. (1985): Influence of rearing medium on size and weight of adults of four *Sitophilus* populations and on weight loss of host kernels (Coleoptera: Curculionidae). Journal of Stored Products Research, 21: 89-93.

Urban J., Lebeda A. 2007. Variation of fungicide resistance in Czech populations of *Psedoperonospora cubensis*. Journal of Phytopathology 155: 143-151.

Urek, G., Milevoj, L. 1993. Ogrčja nadloga: poljedelstvo: varstvo. Kmetovalec, 61: 7-8.

Vet, L.E.M., van Lenteren, J.V., Woets, J. 1980. The parasite-host relationship between *Encarsia formosa* and *Trialeurodes vaporariorum* IX. A review of the biological control of the greenhouse whitefly with suggestion for future research. Zeitschrift für Angewandte Entomology, 90: 26-51.

Weiser, J. 1955. *Neoaplectana carpocapsae* n. sp. (Anguillulata, Steiner-nematidae) novy Cizopasnik housenek obatecejableeneho *Carpocapsa pomonella* L. Vestnik Ceskoslovenske Spoiecnosti Zooloaicke 19: 44-52.

Welch H.E., Briand L.J. 1961. Tests of the nematode DD136 and an associated bacterium for control of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say). The Canadian Entomologist 93, 759-763

White, G. F. 1929: A method for obtaining infective nematode larvae from cultures. Science, 66: 302 – 303

Willmott D.M., Hart A.J., Long S.J., Richardson P.N., Chandler D. 2002. Susceptibility of cabbage root fly *Delia radicum*, in potted cauliflower (*Brassica oleracea* var. *botrytis*) to isolates of entomopathogenic nematodes (*Steinernema* and *Heterorhabditis* spp.) indigenous to the UK. Nematology, 4: 965-970

Zupančič, A. 2008: Research on efficacy of entomopathogenic nematodes (Rhabditida) against cabbage stink bug (*Eurydema ventrale* Kolenati, Heteroptera, Pentatomidae). Graduation Thesis. Ljubljana: XI, 39 pp. [Slovenian]

29

New Mosquito Control Techniques as Countermeasures Against Insecticide Resistance

Hitoshi Kawada
Institute of Tropical Medicine, Nagasaki University
Japan

1. Introduction

Chemical substances, whether naturally occurring or artificially synthesized, continue to play an extremely beneficial role in human life. Life would be unsustainable without the benefits by products of chemical reactions, such as oxygen, water, and various types of nutrient substances. Drugs, foods, fibers, pesticides, etc. have been artificially designed and produced for protecting and improving the quality of human life. Nonetheless, chemical substances have been implicated in the deterioration of human environments and ecosystems. One of the main reasons for this unfair accusation might be inadequate regulations and information due to lack of scientific knowledge based on well founded ecotoxicological and pharmacological researches. Lack of information regarding chemicals has been the major factor that has led to undue credence being given to naturally occurring substances, and social movements, which are propagated by a handful of "fanatics," for resisting artificial chemicals. Biorational and logical approaches by both users and suppliers of chemical substances are therefore required so that humans may continue to benefit from the correct use of chemicals.

Developmental research on pesticides of natural origin is believed to be one of the biorational approaches since it may reduce the adverse environmental impact of chemicals to the level of naturally occurring substances. One of the most successful events in the development of pesticide chemicals was the discovery of pyrethrum and the successful synthesis of pyrethroids. For example, one of the most classical synthesized pyrethroids allethrin (Schechter et al., 1948) continues to be used for preventing mosquito bites without any toxicological and operational problems. The use of pyrethroids for preventing mosquito bites is believed to be biorational because this chemical is safe for mammals. The most popular and long-standing formulations using pyrethroids are mosquito coils, mosquito mats, and liquid vaporizers. Pyrethroids belonging to the knockdown agent group, such as allethrin, pyrethrin, and prallethrin, are used in these formulations. In particular, *d*-allethrin still continues to be used in these types of formulations. Recently, a group of newly developed pyrethroids with high vapor pressure has come to open new era for pyrethroids. Metofluthrin is one of the above promising pyrethroids having high insecticidal activity and high vapor pressure (Ujihara et al., 2004). Metofluthrin belongs to the group of knockdown agents but has a unique

characteristic that none of the conventional pyrethroids possess. The most important unique characteristic of metofluthrin is its high vapor pressure. The vapor pressure of metofluthrin is >2 times and >100 times that of *d*-allethrin and permethrin, respectively, and it vaporizes at room temperature without heating, while other conventional pyrethroids require heating for vaporization. Another unique characteristic is its high efficacy against mosquitoes which is 28–79 times more effective than *d*-allethrin (Argueta et al., 2004). These unique characteristics of metofluthrin may lead to the development of new mosquito controlling devices that do not require any external energy for vaporization and have low cost and longer effective duration.

Pyrethroids belonging to the knockdown agent group have been successfully used worldwide for a long period as a spatial repellent. Spatial repellency will not induce any pyrethroid resistance since it has low lethal activity on the affected insects and causes less selection pressure on insect populations. The discovery of the phenoxybenzyl alcohol moiety accelerated the development of photostable pyrethroids that could be used for outdoor use, including agricultural purposes. These "second generation" pyrethroids have been used worldwide as good vector control agents with various application techniques, such as residual spraying, ULV spraying, and long lasting insecticide-treated net (LLITN). However, photostable and highly effective pyrethroids might accelerate the development of pyrethroid resistance in mosquito populations. Photostable pyrethroids consist of 2 structurally different types of chemicals according to the presence of α-cyano moiety, type I (permethrin, etofenprox, etc.) and type II (deltamethrin, lambda-cyhalothrin, cypermethrin, etc). Olyset® Net is one of the most promising LLITN. Olyset® Net is slow-releasing formulation composed of plastic fibers impregnated with permethrin—one of the most popular and safe type I pyrethroids. Recently, Siegert et al. (2009) reported that the Olyset® Net reduced landing attempts of mosquitoes and elevated their flight frequency, resulting in little mortality, while mosquito landing attempts on the PermaNet®, containing type II pyrethroid, deltamethrin, under the same conditions were sustained longer and caused greater mortality than the Olyset® Net. This appears to be important for an effective control of the mosquito population. The highly lethal pyrethroids with less excito-repellency appear to be most effective for reducing vector mosquito population. Such highly lethal pyrethroids, however, might accelerate the development of resistance. The exito-repellency of slow-released permethrin, on the contrary, might reduce the human—vector contact and blood feeding success. In fact, there was no difference between Olyset® Net and PermaNet® in the field efficacy as measured by blood feeding rate (Dabire et al., 2006). The positive use of exito-repellency of slow-released pyrethroids, therefore, might lead bio-rational vector control with the maximum reduction of mosquito biting and minimum risk of resistance.

Juvenile hormone mimics (JHMs), which have also been developed from natural sources, are among the most studied and effective chemicals, and are categorized as insect growth regulators (IGRs). These chemicals have a unique mode of action that is insect-specific, stage-specific, slow acting, and not neurotoxic (Miyamoto et al., 1992). Methoprene (Henrick et al., 1973) and pyriproxyfen (Hirano et al., 1998) are the most successful JHMs. Almost 40 years have passed since Williams (1967) suggested that JHMs could be the 3rd generation insecticides that will not adversely affect the ecosystem due to their target-specificity, and against which pests theoretically have no potential of developing resistance. However, the above beliefs have been proved incorrect or have changed during the 3 decades since the first successful JHMs methoprene and hydroprene were

commercialized. Insect resistance to JHMs has become common among agricultural and non-agricultural pests (Zhang et al., 1998; Cornel et al., 2002; Ishaaya et al., 2005), and several reports have demonstrated that JHMs may adversely affect the ecosystem if they are overdosed (Miyamoto et al., 1993; Trayler and Davis, 1996). Therefore, utmost care and high level expertise are a requisite for the biorational use of JHMs, and application of the minimum dose in the most effective manner will result in maximum benefits both to humans and the ecosystem.

In this chapter, several attempts to develop new mosquito control techniques with using a pyrethroid belonging to knockdown agent groups (metofluthrin) and a slow-released type I pyrethroid (permethrin) as a spatial and exito-repellent agent are introduced. The new biorational use of JHM (pyriproxyfen) as a "mosquito population growth regulator" is also discussed.

2. Field evaluation of spatial repellency of metofluthrin-impregnated plastic strips against vector mosquitoes

Metofluthrin (SumiOne®), 2,3,5,6-tetrafluoro-4-methoxymethylbenzyl(E:Z ≈1:8)(1*R*, 3*R*)-2,2-dimethyl-3-(prop-1-enyl)cyclopropanecarboxylate, is a newly synthesized pyrethroid. The high knockdown and lethal activity of metofluthrin against mosquitoes has been demonstrated previously. The high vapour pressure of metofluthrin (1.87×10^{-3} Pa at 25˚C), which is 2-fold and 100-fold greater than those of *d*-allethrin and permethrin, respectively, enables vaporization at normal temperature in the absence of heating, while the other conventional pyrethroids require heating for evaporation. The unique characteristics of metofluthrin may lead to the development of novel mosquito-controlling devices that require no external energy for vaporization and those that provide long-term efficacy at low maintenance costs.

In preliminary studies, using a simple prototype device with metofluthrin-impregnated multilayer paper strips, the chemical showed promising spatial repellency against mosquitoes in both laboratory and field conditions (Kawada et al., 2004a; 2004b). Under simulated outdoor conditions, mosquitoes (*Anopheles* sp. and *Culex* sp.) were repelled by airborne metofluthrin vapours (Fig. 1, 2; Kawada et al, 2004a). The field tests suggested that metofluthrin may be a good candidate for the prevention of mosquito bites (*Anopheles sundaicus* (Rodenwaldt), *Anopheles balabacensis* (Baisas), and *Culex quinquefasciatus* (Say)) in shelters without walls (beruga), those used by people in Lombok Island, Indonesia and which are associated with high risk of malaria transmission (Kawada et al., 2004b). In order to increase the effectiveness of metofluthrin, Kawada et al. (2005a) manufactured a cylindrical slow-release plastic formulation that was impregnated with 1000 mg metofluthrin in a 20 g strip. By using this formulation, the authors obtained prolonged duration of activity (>14 weeks at the rate of 4 strips per beruga) in the beruga under outdoor conditions in Lombok (Fig. 3,4).

Further, spatial repellency of this plastic formulation against *Aedes aegypti* (L.) was achieved in the residential houses of Do Son, Hai Phong city, Vietnam (Kawada et al., 2005b). The above study has confirmed the long-lasting spatial repellent efficacy of metofluthrin-impregnated plastic strips against *Ae. aegypti* under indoor conditions; however, the effective duration (6 weeks at 1 strip per room) appeared insufficient for practical use (Fig. 5).

Fig. 1. Prototype Multilayer paper strip device impregnated with 200 mg of metofluthrin and outdoor human-baited collection with a double net (Strips were hung in a space between the inside and outside nets) (Kawada et al., 2004a).

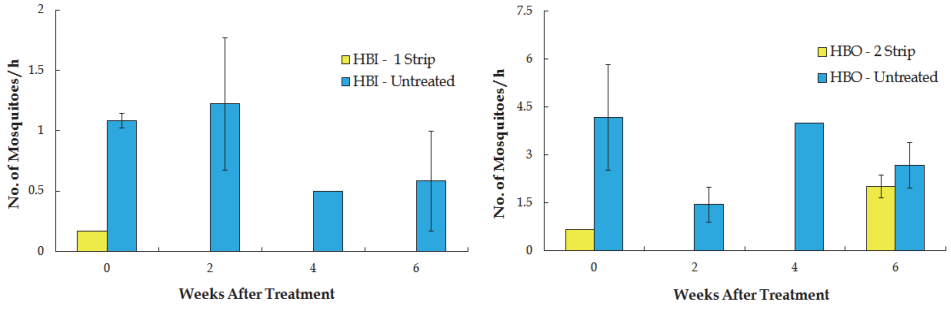


Fig. 2. Changes in total number of mosquitoes collected per hour at indoor human-baited collection (HBI) and outdoor human-baited collection (HBO) after the treatment with multilayer paper strip device impregnated with metofluthrin (Kawada et al., 2004a).

Fig. 3. Metofluthrin-impregnated plastic strip for the trial and the field test scene with the strips in a beruga where Lombok people spend every evening before going to bed (Kawada et al., 2005a).

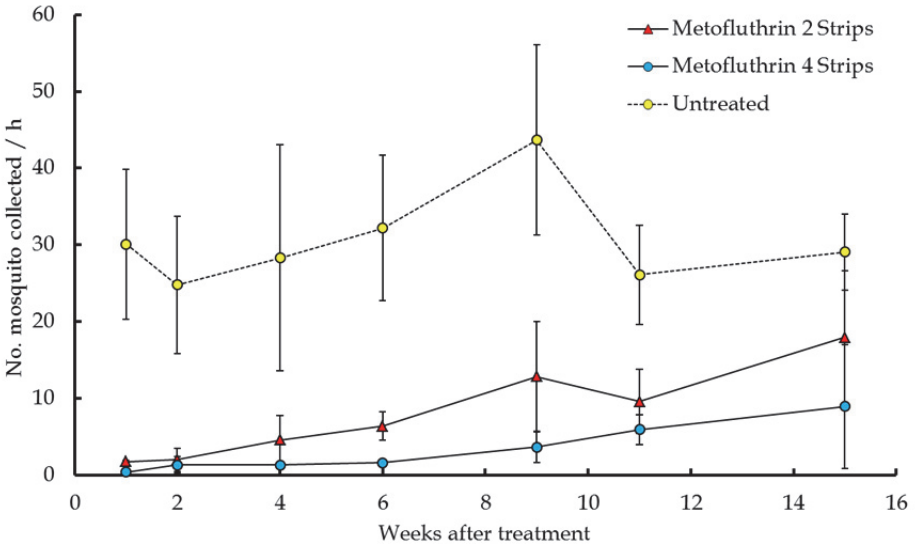


Fig. 4. Changes in the total number of mosquitoes collected per h during the trial for metofluthrin-impregnated plastic strips. Bars indicate the standard deviations (Kawada et al., 2005a).

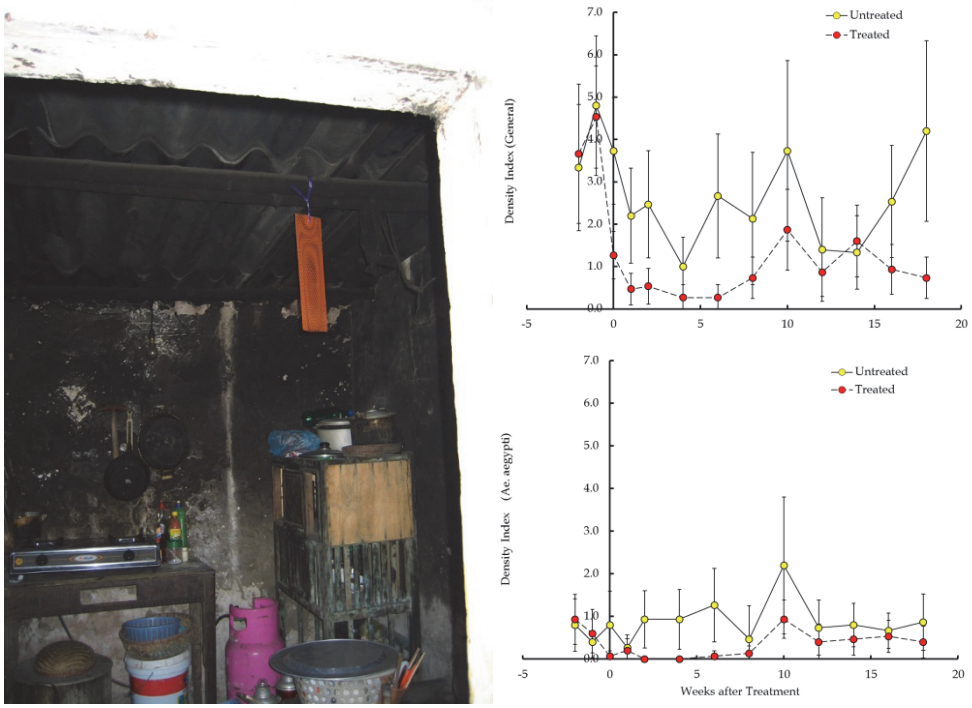


Fig. 5. Treatment scene with a metofluthrin-impregnated plastic strip in a room, Do Son, Vietnam (Left photo) and changes in the number of mosquitoes collected in metofluthrin-treated and untreated houses (Right graphs). Upper graph shows the general density index based on the total number of mosquitoes collected (*Cx. quinquefasciatus* and *Ae. aegypti*) and lower graph for *Ae. aegypti* index (Kawada et al., 2005b).

Long-term effectiveness of these devices may be achieved by using the following methods: (1) designing devices of different shapes, (2) adopting formulations with different optimal compositions and densities of the plastic polymer in order to reduce the release rate of the active ingredient, (3) increasing the concentration of the active ingredient, and (4) increasing the number of strips per room. Accordingly, as the next step in the development of the devices, a new latticework plastic strip that was designed to reduce the release rate of metofluthrin to approximately 50% of that obtained in the previous plastic formulation was manufactured. The new latticework strips (approximately 600 mg metofluthrin per 12.3 g strip) at 1 strip per 2.6–5.5 m² were effective for at least 8 weeks against *Ae. aegypti* in the residential houses in My Tho city, Tien Giang province, Vietnam (Fig. 6,7; Kawada et al., 2006a).

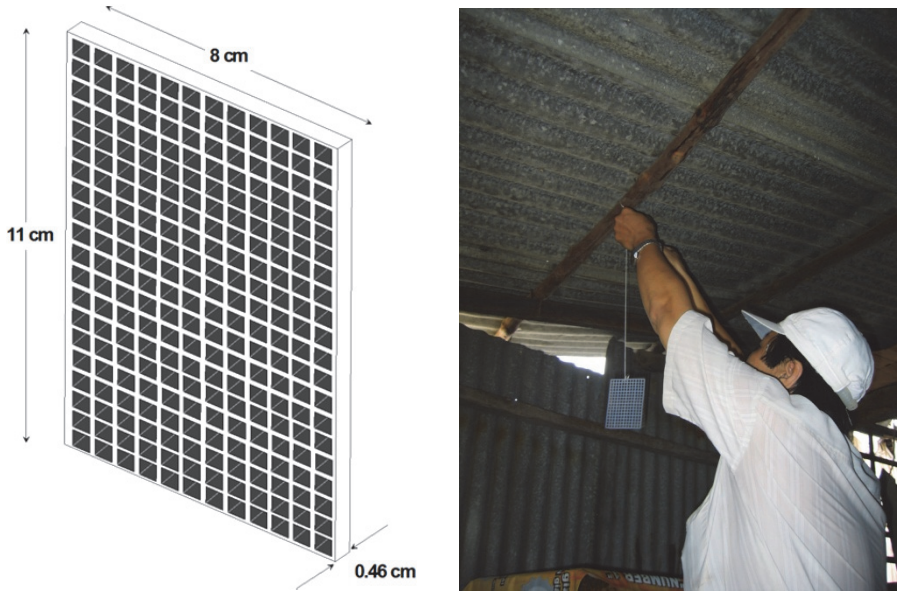


Fig. 6. Metofluthrin-impregnated polyethylene latticework strip (left) and treatment scene of the strip in a room (right), My Tho city, Vietnam (Kawada et al., 2006a).

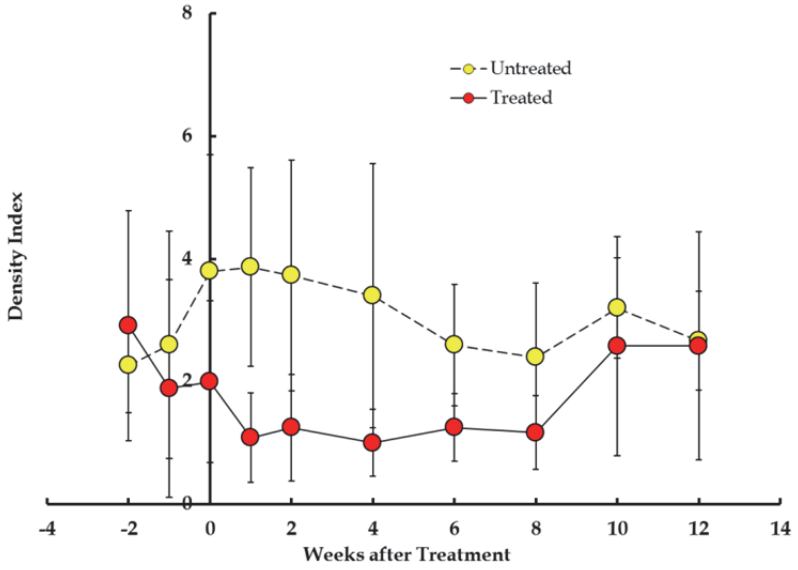


Fig. 7. Changes in the mosquito density index (female per house per day) of *Aedes aegypti* collected in metofluthrin-treated and untreated houses, My Tho city, Vietnam. Bars indicate 95% confidence limits (Kawada et al., 2006a).

The above new prototypes of metofluthrin-impregnated latticework plastic strips were evaluated against malaria vector, *Anopheles gambiae* Giles complex, in the Kongo villages of Bagamoyo district in coastal Tanzania (Kawada et al., 2008). The study using 20 houses, half intervention, half control, were conducted for 124-day period. Pyrethrum spray sheets collection and CDC light traps were used to sample mosquito population indices. The mosquito density indices of the intervention houses were observed to be significantly lower than those of the control houses when pyrethrum spray sheet collection was used (Fig. 8 and Table 1; F = 4.61, 1 df, P = 0.038; 98.7% reduction of total mosquito collection compared with that for the controls). These low indices were observed despite the large opening area of Bagamoyo houses that were considered to have a considerable negative effect on the spatial repellency of metofluthrin.

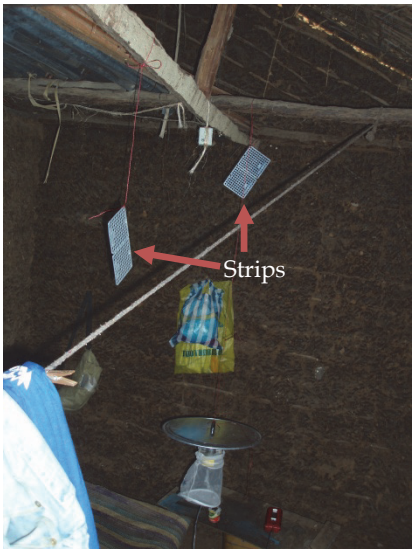


Fig. 8. Treatment scene of metofluthrin-impregnated latticework plastic strips in a room, Bagamoyo, Tanzania. CDC light traps were put inside rooms for collection of mosquitoes (Kawada et al., 2008).

Days after Intervention	Mosquito Density Index (*Anopheles gambiae* s. l.)[1)]			
	Intervention - (95%CI)		Control - (95%CI)	
20	0.2	(0.4)	2.0	(1.0)
34	0.2	(0.4)	11.4	(5.6)
61	0.0	(-)	8.0	(4.2)
89	0.0	(-)	7.2	(4.4)
124	0.0	(-)	2.4	(2.3)

[1)] Mosquito Density Index = No. of female mosquito / house / day

Table 1. Changes in the mosquito density index by pyrethrum spray sheet catch collection in the metofluthrin-intervention and control houses in Bagamoyo, Tanzania.

Table 2 lists the environmental factors and the effective duration of the metofluthrin-impregnated plastic strips in the present study as well as those of the intervention houses measured in My Tho city, Tien Giang, Vietnam, where a similar metofluthrin trial was conducted in the same season in the year 2005 (Kawada et al. 2006a). Variables including the average temperature and humidity were calculated on an hourly basis from June 20 to August 3, 2006 for Bagamoyo and from June 20 to September 4, 2005 for My Tho. The room temperature was lower and the humidity was higher in Bagamoyo houses compared to the corresponding conditions in the My Tho houses. Although the floor area and the volume were larger in the houses in My Tho compared to those in Bagamoyo, the corrected opening area per total average volume of the houses in Bagamoyo was almost twice that of houses in My Tho, thereby indicating that the Bagamoyo houses are more "open" than the My Tho houses (Kawada et al., 2008).

Environmental Factors	Trial Sites (Trial Year) - Target Mosquito			
	My Tho (2005)[2)] *Ae. aegypti*		Bagamoyo (2006)[2)] *An. gambiae* s.l.	
Average Temperature (°C)[1)]	29.1	(0.8)	24.8	(0.7)
Average Humidity (% RH)[1)]	70.1	(5.1)	75.3	(3.9)
Total Floor Area (m^2) / House	32.1	(10.5)	22.0	(14.1)
Total Volume (m^3) / House	129.3	(59.4)	58.7	(45.7)
Total Opening Area (m^2) / House	6.6	(5.0)	5.7	(4.3)
Corrected Opening Area / Volume	0.051		0.098	
No. of metofluthrin Strips / m^2	0.31		0.52	
Amount of metofluthrin (mg) / m^2	191		320	
Effective Duration (Weeks)	8		> 18	

[1)] June 20 - August 3, 2006 in Bagamoyo; June 20 - September 4, 2005 in My Tho
[2)] Figures in parenthesis are standard deviations

Table 2. Environmental factors of the intervention houses and effective duration of metofluthrin-impregnated plastic strips.

Metofluthrin-impregnated strips significantly reduced the density index of mosquitoes in the intervention houses in several different environmental conditions in Indonesia, Vietnam, and Tanzania. Kawada et al. (2004a, 2004b, 2005a, 2005b, 2006, 2008) reported that mosquitoes were repelled by airborne metofluthrin vapors due to the two main modes of pyrethroid action, i.e., knockdown activity and biting inhibition or disruption of orientation toward the host. Of these, the latter may be categorized as a sublethal and "delayed" effect that results from neural excitement, which appears to occur at an earlier stage of pyrethroid toxicity (MacIver 1964, Winney 1975, Birley et al. 1987). Kawada et al. (2006a) reported that both the increase in the average room temperature and the decrease in the opening area of the rooms treated with metofluthrin-impregnated strips exerted an increased spatial repellent effect. The increase in temperature might increase the evaporation rate, and the decrease in the opening area might retain the active ingredient inside the rooms thereby resulting in an increased concentration of metofluthrin in the air. The corrected opening area/volume in the houses in Bagamoyo was nearly twice as much as that of the houses in My Tho city (Table 2). A large opening area would potentially facilitate the entry of

endophilic and nocturnal mosquitoes, and the presence of large and numerous open eaves in the typical rural African houses are considered to be one of the most important entrances for invasion by *An. gambiae* during the night. Snow (1987) reported that the invasion by *An. gambiae*, *Anopheles melas* Theobald, and *Mansonia* sp. into the experimental huts was slightly affected by increasing the wall height. Lindsay et al. (2003) reported that the entry of *An. gambiae* into house was reduced by 37% subsequent to the closure of the eaves. Similarly, a significant contribution of open eaves to the increase in mosquito invasion was reported by Pålsson et al. (2004). We, therefore, argue that the large opening area of the houses in Bagamoyo might have negatively affected the spatial repellent efficacy of metofluthrin. The effective duration of repellency (>18 wk) is believed to be sufficient for the practical application of these devices considering the convenient replacement of the formulation. Further improvements related to the manufacturing of plastic strips, such as optimization of the composition and the density of the plastic polymer in order to reduce the loss of the active ingredient, may enable the development of an optimum formulation that would result in a longer effective duration and lower treatment cost.

3. Preventive effect of release controlled plastic net of permethrin (Olyset® Net) against vector mosquitoes

The use of insecticide-treated bed nets (ITNs) as a simple and inexpensive self-protection measure against malaria has been shown to reduce morbidity of children (< 5 years old) by 50% and global child mortality by 20%–30% (Binka et al., 1996; Lengeler et al., 1996; Nevil et al., 1996). Impregnation and the re-impregnation of ITNs, however, needed technical skills, materials, and human costs which may not always be available (Lines, 1996). The mosquito nets pre-treated with insecticide and with longer lasting effect (LLITNs) were one of the break-through measures to this problem (Guillet et al., 2001). Olyset® Net, made of polyethylene netting material (mesh 20 holes/cm^2) with permethrin (2%) incorporated into the polymer before monofilament yarn extrusion, and the PermaNet®, made of polyester netting material (mesh 25 holes/cm^2) with deltamethrin (55 mg ai/m^2) incorporated in a resin coating of the fibers, are two successful products among the LLITNs which WHO had recommended.

Several attempts to apply the LLITNs to the other vectors, such as *Ae. aegypti* (Curtis et al., 1996; Igarashi, 1997; Kroeger et al., 2006; Jeyalakshmi et al., 2006) and *Phlebotomus* (Dinesh et al., 2008; Faiman et al., 2009; Emani et al., 2009; Kasili et al., 2010; Das et al., 2010), have been performed and trials to apply the LLITNs as the other controlling tools, such as curtains (Curtis et al., 1996; Igarashi, 1997; Kroeger et al., 2006; Vanlerberghe et al., 2011a; 2011b) and jar covers (Kroeger et al., 2006; Vanlerberghe et al., 2011a; 2011b), have been reported using Olyset® Net and/or PermaNet®. In this section, a new attempt for controlling *Ae. aegypti* using Olyset® Net as water jar covers is introduced and a new self-protection technique for preventing malaria vectors using Olyset® Net materials are proposed.

3.1 Effect of release controlled plastic net of permethrin (Olyset® Net) as water container cover on field populations of *Aedes aegypti* in Southern Vietnam

Dengue fever first appeared in Vietnam at Hanoi and Haiphong in 1959 and since then has become endemic throughout the whole country (Nam et al., 2000). Jars, tanks, and drums provide suitable breeding sites for *Ae. aegypti* in Vietnam (Phong and Nam, 1999; Nam et al., 2000; Tsuzuki et al., 2009) and these breeding sites are important targets for controlling

immature stages of *Ae. aegypti*. Insecticide treatment to such breeding sites with organophosphates such as temephos or insect growth regulator (IGRs) such as pyriproxyfen, both of which are recommended to treat in drinking water by WHO, seems to be best and most convenient measures. However, treatment of any insecticide to such breeding sites is legally prohibited in Vietnam, making the larval vector control more difficult. Preventing invasion of gravid female mosquitoes into the above breeding sites is also important as well as removing these habitats.

Tan Chanh, a commune of Long An province, located 30 km south from Ho Chi Minh City, Vietnam was selected as trial site. Release controlled plastic net of 2% permethrin (Olyset® Net) and EcoBio-Block® S, a novel release controlled system for the insect growth regulator, pyriproxyfen, composed of a porous volcanic rock and cement and which incorporated the aerobic bacteria groups of *Bacillus subtilis natto* as a water purifying agent (Kawada et al., 2006b) were used for the study. Residential colony of Tan Chanh was assorted into 20 adjacent clusters which contain each 50 houses. Among them, ten clusters were selected as trial site for intervention by Olyset® Net and EcoBio-Block® S and other 10 clusters as control. For trial site, all water jars were covered with lids equipped with Olyset® Net. Olyset® Net was cut in 30 × 150 cm and tied along the circumference of a lid (Fig. 9). Additionally, all the other breeding containers such as flower vase inside and peripheral of houses were treated with EcoBio-Block® S, crushed into small pieces (less than 1 cubic cm) and were put in the container at the rate of approximately 1g per 1 litter of water.

Fig. 9. Olyset® Net was cut in small pieces (Left Photo) and tied along the circumference of jar covers (Right Photo).

House index (Percentage of houses or premises positive for *Aedes* larvae) in Olyset® Net treated area was higher than that of control before intervention and the index sharply decreased during one month after intervention. The house index gradually increased from October, 2008 to February, 2009, while it kept lower value than that in untreated area. Container index (percentage of water holding containers positive for *Aedes* larvae) also decreased in the same way as in the house index in the Olyset® Net treated area and kept lower level at least for 5 months after intervention (Fig. 10).

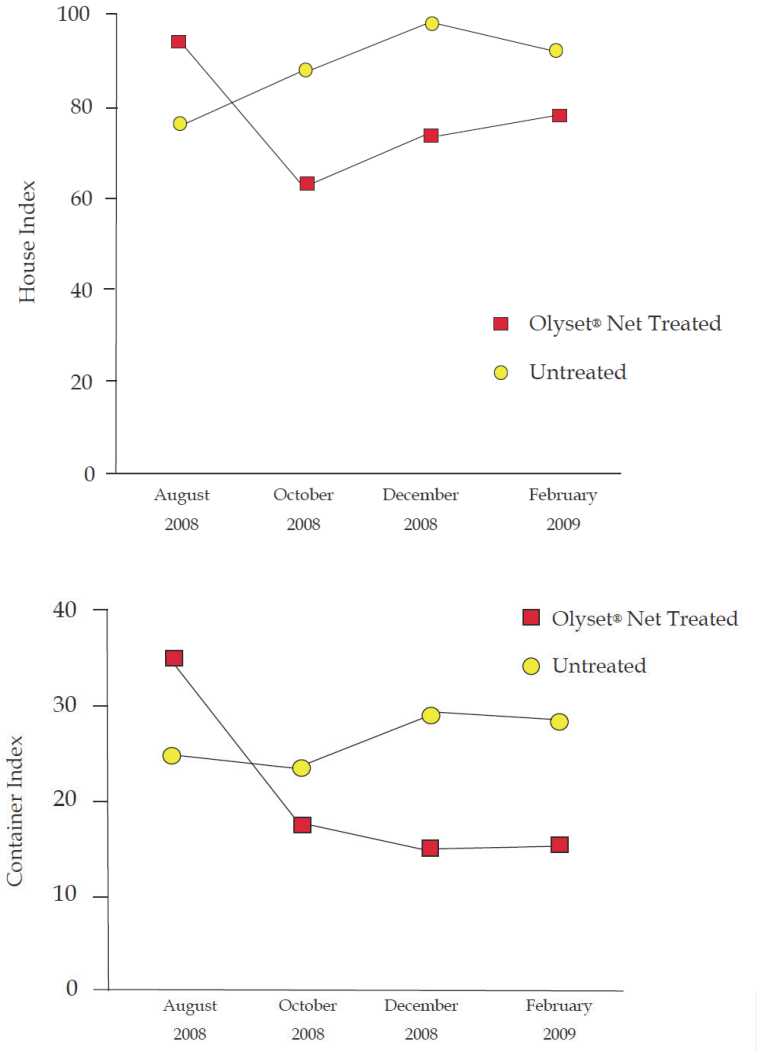


Fig. 10. House index in Olyset® Net treated and untreated areas in Tan Chanh, Vietnam from August 2008 to February 2009. Intervention was carried out in September, 2008 (Tsunoda et al., 2011).

3.2 Small scale experiment of a house screening technique using Olyset® Net material in malaria endemic area in Kenya

Use of ITNs or LLITNs is effective against malaria vectors when the vector mosquitoes are endo-phagous and their feeding time corresponds to the time when people are sleeping inside bed net. Behavioral resistance, such as behavioral change in vector mosquitoes from endo-phagous to exo-phagous and/or shifting of biting time from midnight to dawn or dusk, may reduce the effectiveness of bed nets as well as physiological resistance to insecticides. Most important limitation for the effective use of bed net is that it is only effective when people are sleeping inside. Recently, Iwashita et al. (2010) reported that bed net use by children between five and 15 years of age in villages along the Lake Victoria, western Kenya was lower than that among the other age classes. Bed net use was strongly affected by sleeping arrangement and availability of suitable locations for hanging bed nets. The easiness of hanging a bed net is particularly important for children who often are sleeping in the other place such as living room where the net hanging is difficult. Daily hanging of bed nets in the above place might be troublesome for residents. Hence, the uses of bed net are sometimes limited to the persons sleeping in a bedroom (parents and babies) and the rest of family members (ex. children > 5 years old) are found to sleep in living room with no bed net, resulting in the high Pf positive case in these generations. Therefore, new devices which can substitute bed nets or new self-protection measures which are convenient and sustainable for residents is required for the more effective prevention of malaria vectors.

On the other hand, eaves, the gaps between the top of the wall and the roof, are one of the most common house structures in Africa and are thought to be the most important entrance for malaria vectors (Njie et al., 2009). Changes in house design may reduce human exposure to malaria vectors. Screening or closing eaves was reported to be effective (Lindsay et al., 2003). Restructuring of houses or physical closing the eaves, however, will require much cost and cause deterioration of living environment by blocking ventilation. Net screening of ceilings and eaves is likely to be well accepted and of greatest benefit to moderate disease transmission (Lindsey et al., 2003; Kirby et al., 2009). Use of nets with coarse mesh size will be most acceptable in considering the good ventilation.

Small scale trial using Olyset® Net materials were performed in Mbita, Nyanza province, western Kenya in 2010 and 2011. *Anopheles gambiae* s.s., *Anopheles arabiensis*, *Anopheles funestus* s.s. are main malaria vectors in this area. *Anopheles rivulorum*, which is one of the sibling species in *An. funestus* complex, is also minor vector in this area. The above three main vectors were recently reported to have developed multimodal pyrethroid resistance (Kawada et al., 2011a).

The Olyset® Net materials impregnated with 2% permethrin was used in the study. The net materials were cut and sewed into a 7 x 5 m sheet and ring bands were equipped on the diagonal position of the nets to ease the fixation of nets under ceiling (Fig. 11, 12). The study was performed in three houses (two houses for intervention, the other one house for control) in Nyandago village in Gembe East, Mbita Division in the Suba district of the Nyanza province, western Kenya. The shielding effect of the ceiling nets was evaluated by the number of indoor resting mosquitoes collected using the battery-powered aspirator.

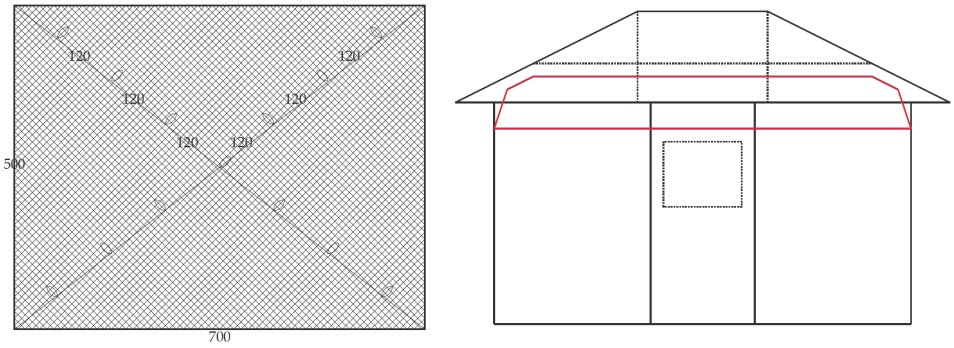


Fig. 11. Ceiling net using Olyset® Net materials (Left) and outline sketch of the ceiling net installed in a house (Right) (Kawada et al., 2011b).

Fig. 12. Intervention scene of permethrin-impregnated ceiling net (Kawada et al., 2011b).

Olyset® Net experimentally used for covering the ceiling and closing eaves, in the present study, resulted in outstanding reduction of the number of resting mosquitoes inside houses. The number of mosquitoes drastically decreased 1 day after the intervention of ceiling nets and lower densities were kept for 9 months until the removal of the nets, while the mosquito density in the control house kept high level during the above period (Fig. 13). Lindsay et al. (2003) reported that little difference in the protecting effect of insecticide-treated and untreated screen nets. The present study, however, emphasizes the necessity of the presence of insecticide impregnated nets as a chemical barrier, which may partly be due to the coarse mesh size of Olyset® Net materials to ease the ventilation (Fig. 14). Screening of ceiling and closing eaves with insecticide-treated nets with coarse mesh size such as Olyset® Net will be acceptable way to residents and effective interfering measure for preventing the mosquito entering in houses with small cost and minimum environmental deterioration.

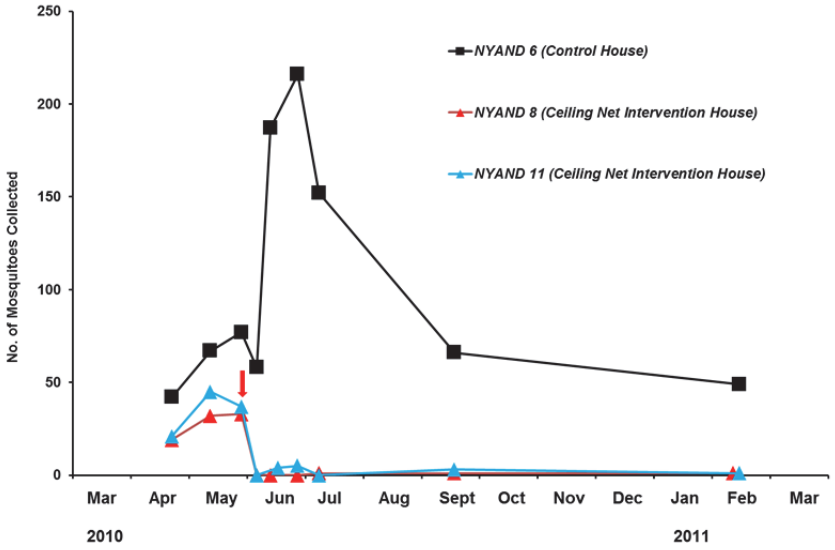


Fig. 13. Changes in the number of mosquitoes collected in the ceiling net intervention houses (NYAND 8, 11) and control house (NYAND 6). Red arrow indicates the day of intervention (Kawada et al., 2011b).

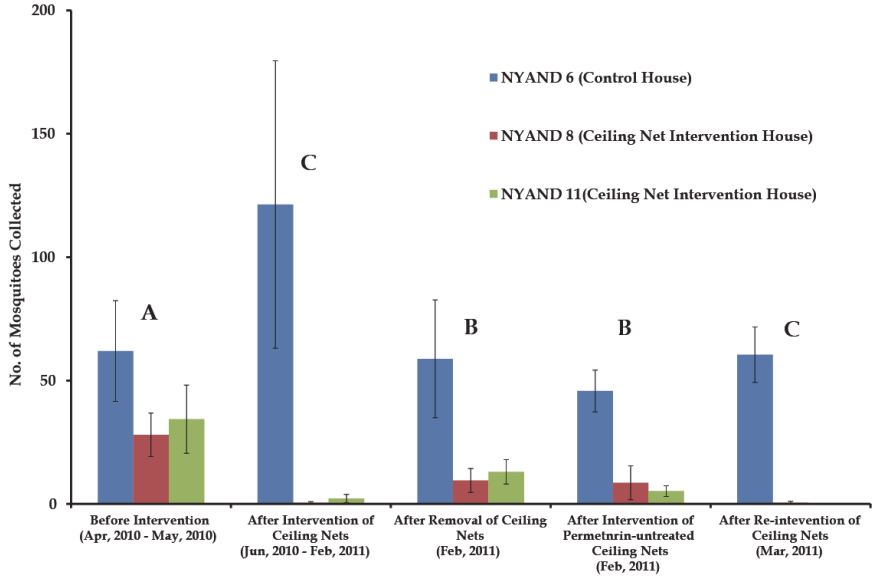


Fig. 14. Average number of mosquitos collected before intervention of permethrin-impregnated ceiling nets, after intervention, after removal of the permethrin-impregnated

ceiling nets, after intervention of permethrin-untreated ceiling nets, and after re-intervention with new permethrin-impregnated ceiling nets. Bars indicate 95% confidential limits. The same letters indicate no significant difference when square root of the ratio of the number of mosquitoes collected in the intervention house versus that collected in the control house was converted into Arcsin and the multiple comparison of the ratio was performed by Tukey's HSD test (P = 0.05) (Kawada et al., 2011b).

4. Effect of Juvenile Hormone Analogue (JHM), pyriproxyfen, as a mosquito population growth regulator

From a medical point of view, mosquitoes are thought to be the most important order of insects. Larviciding seems to be the most suitable measure for controlling mosquitoes, since larval habitats are often limited in small and/or local area. Most of the effect of JHMs is on the last instar larvae which becomes deformed or dies at pupal stage as a result of the treatment (Hirano et al., 1998). Pyriproxyfen was observed to cause vacuolation and inhibition of development of imaginal buds of *Ae. aegypti* larvae, and histolysis, such as disrupted mitochondria, abundant vacuoles and poorly-structured cytoplasmic organelles were also observed (Syafruddin et al., 1990). Adult *Anopheles balabacensis*, which survived 48 hr of immersion in 0.005 ppb (one eighth of LC_{50}) of pyriproxyfen during their last larval instar, was found to show considerable reduction in sperm and egg production and also in blood feeding and mating activity (Iwanaga and Kanda, 1988).

JHMs act as "sterilant" when it was treated to adult insects. Methoprene was reported to affect ovarian development and adult longevity in *Ae. aegypti* (Judson and Lumen, 1976; Klowden and Chambers, 1989). The number of eggs/female and hatchability decrease with the application of pyriproxyfen to female *Ae. aegypti* (Kawada et al., 1993; Itoh et al., 1994). Additionally, when a blood-fed female of *Ae. aegypti* had been in contact with pyriproxyfen, a quantity of pyriproxyfen was transferred from her body to the water adjacent where she laid her eggs (Kawada et al., 1993; Itoh et al., 1994). Significant inhibition of emergence was observed in the field trial in Bangkok, where "resting traps" inside of which was treated with oil formulation of pyriproxyfen were placed in a room (Itoh, 1994). Recently, several semi-field trials on the control using the horizontal transfer of pyriproxyfen by *Ae. aegypti* females to their breeding sites were reported to be successful (Shihuincha et al., 2005; Devine et al., 2009).

Ohba et al. (2011) reported the usefulness of pyriproxyfen-treated net for the control of *Aedes albopictus*. Two microcosm trials using the polyethylene net treated with 0.1% and 1% of pyriproxyfen were conducted (Fig. 15). Laboratory colony of *Ae. albopictus* was released into microcosms as shown in Fig. 15 and were allowed to feed on a mouse in a pyriproxyfen treated or untreated net cage (50×50×50 cm) on which small holes (ϕ 5 cm) were placed in order to the female mosquitoes could contact the nets when they flew into the cage for blood feeding. After experimental colony of *Aedes albopictus* was released into the microcosm containing pyriproxyfen treated nets, the number of eggs oviposited and the number of pupae were significantly lower compared with untreated controls (Fig. 16). Egg hatchability of the eggs oviposited by pyriproxyfen-exposed females was significantly suppressed and horizontal transfer of pyriproxyfen by females was also observed by bioassay using the water in which the above females oviposited.

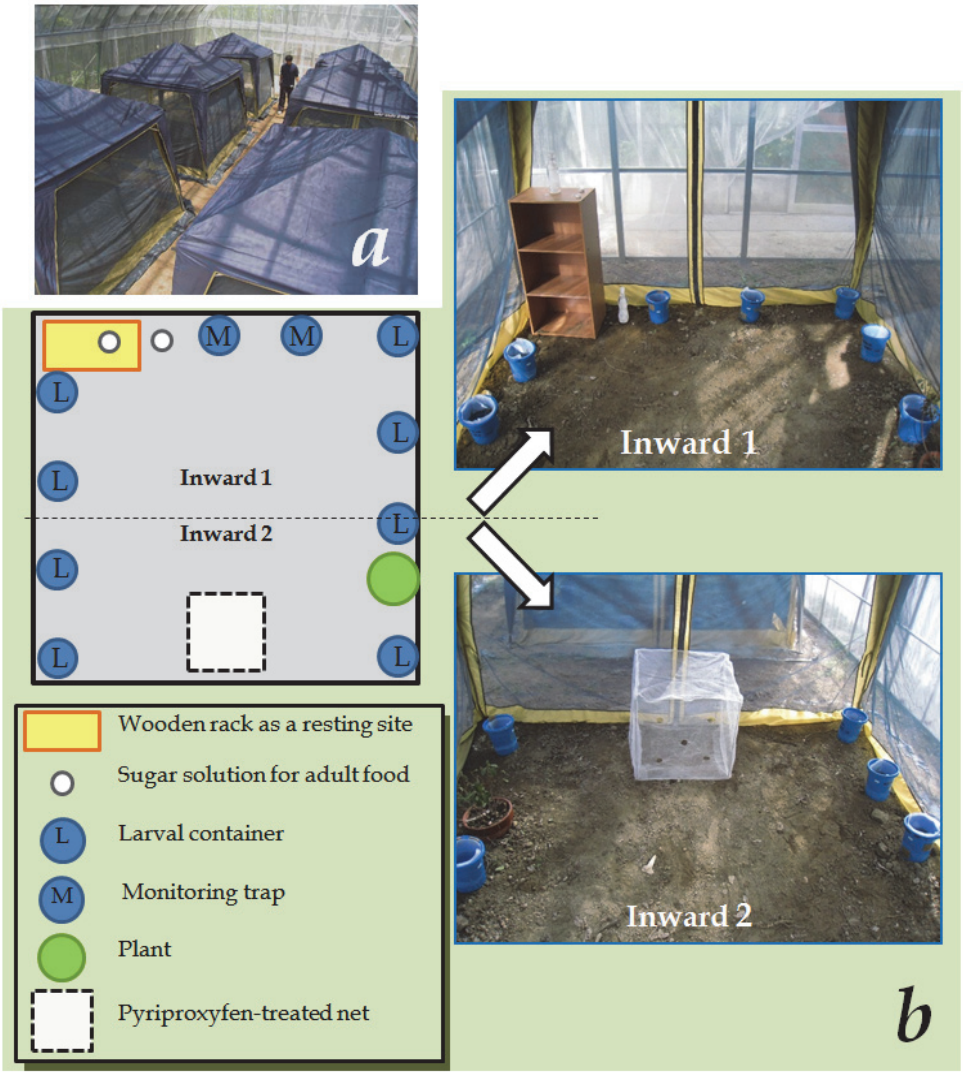


Fig. 15. Diagram and photographs of the semi-field experiment. a, overall view of the greenhouse where the tents (simulated microcosm) were located; b, diagram of the microcosm. Larval container, eight ovitraps were put counting the number of eggs and pupae oviposited; Monitoring trap, two ovitraps were put for the observation of 1) egg hatchability, 2) larval bioassay for the emergence inhibition of pyriproxyfen transferred by adult females. Three microcosms were installed with pyriproxyfen-treated nets and the other three were for untreated nets (Ohba et al., 2011).

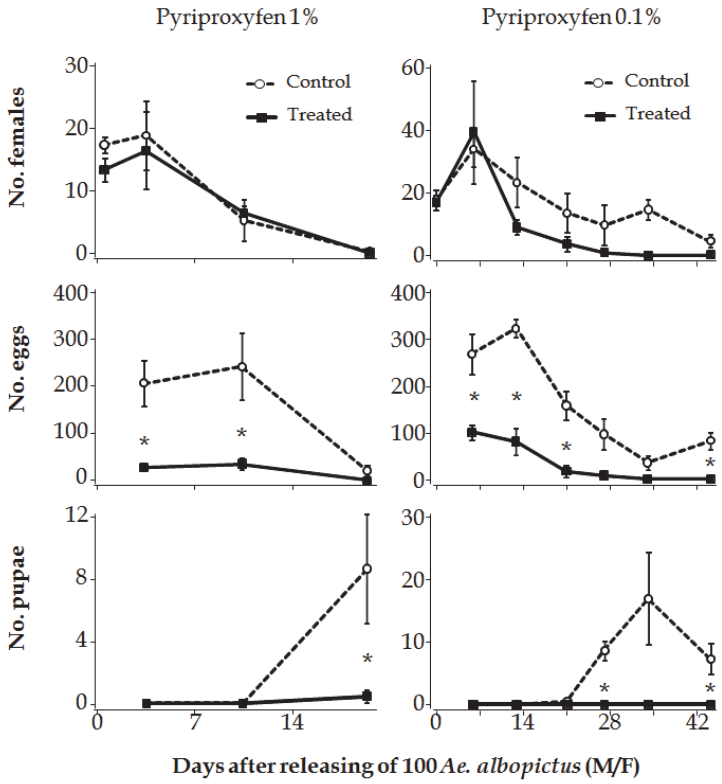


Fig. 16. Effect of pyriproxyfen on the number of females, number of eggs oviposited, and number of pupae in the microcosms in which *Ae. albopictus* females were allowed to contact pyriproxyfen-treated nets (* difference is significant at $P < 0.05$, one-way ANOVA) (Ohba et al., 2011).

5. Conclusion

Insecticides still provide the most promising countermeasures for controlling malaria, dengue hemorrhagic fever (DHF), and other arthropod-borne diseases. On an average, at the global level, > 500 tones of DDT, ca. 40 tones of organophosphates, ca. 20 tones of carbamates, and ca. 40 tones of pyrethroids are used as active ingredients annually for indoor residual spraying against malaria vectors (Zaim and Jambulingam, 2007). The average total amount of pyrethroids used annually as active ingredients between 2003 and 2005 at the global level was 161 tones, which is 16% of the total insecticide consumption and 36% of the total insecticide consumption if the amount of DDT, which is exclusively used in African countries, is excluded. Among pyrethroids that are used for vector control, 98.7% comprise photo-stable pyrethroids such as α-cypermethrin, bifenthrin, cyfluthrin, cypermethrin, deltamethrin, etofenprox, λ-cyhalothrin, and permethrin (Zaim and Jambulingam, 2007). Pyrethroid resistance will be a major problem for the vector control program, since at present, there are no suitable chemical substitutes for pyrethroids.

Vu et al. (2004) conducted WHO standard bioassay using adult *Ae. aegypti* collected in 22 places in 11 provinces and cities in four different regions of Vietnam and found that the mosquitoes were susceptible to pyrethroids in many places in the North and Centre regions but they were resistant in the South and Central Highlands in Vietnam. Kawada et al. (2009a) reported similar tendency in pyrethroid susceptibility in *Ae. aegypti* in Vietnam, and found that new *kdr* mutations on domain III were widely distributing in southern Vietnam (Kawada et al., 2009b). The above authors concluded this discrepancy in pyrethroid susceptibility in different regions to be due to the longer and extended use of pyrethroids in malaria and dengue fever control programs and in agriculture in the Southern and Central Highlands. Actually, a lot of pyrethroids have been treated as residual treatment inside houses and pyrethroid-impregnated bed nets for malaria control as a part of the National Malaria Control Program. The pyrethroid use for malaria control seems to be important factor in developing pyrethroid resistance in *Ae. aegypti* in highland region of Vietnam, since the DF/DHF cases are not serious and forest malaria continues to be endemic in this region as compared to the other regions and consequently the amount of pyrethroid treatment for dengue vector control in highland region is lower than the other regions. The pyrethroid treatment for malaria vector control appears to have been intensively conducted in the interior and along the periphery of human habitation areas, where incidentally, the breeding and resting sites of *Ae. aegypti* are located and this might account for the strong selection pressure toward *Ae. aegypti* (Kawada et al., 2009). In Vietnam, 24 tonnes of DDT was used for residual treatment against malaria vectors in 1993 and 1994. However, since the abandoning of DDT sprays in 1995, only pyrethroids (residual spraying of λ-cyhalothrin and α-cypermethrin and occasionally deltamethrin, and permethrin-impregnated bed nets) have been extensively used in large amounts, unlike in the other Asian countries (Nam et al., 2005; Zaim and Jambulingam, 2007). Although details regarding the amount of insecticides used for dengue control in Vietnam have not been published, 21,000 liters of photo-stable pyrethroid formulations such as λ-cyhalothrin, deltamethrin, and permethrin was reported to be used for dengue control in 20 southern provinces in 2007 (Epidemiological and virological vector surveillances for dengue control program in southern Vietnam, 2008, Pasteur Institute, Ho Chi Minh City). The extensive use of photo-stable pyrethroids, therefore, seems to have been very common in southern Vietnam.

On the other hand, in Kenya, the high allelic frequency of *kdr* mutations (L1014S) in both *An. gambiae* s.s. and *An. arabiensis* were reported to convergently distribute in western part including highland region as well as northern and southern coastal region of Lake Victoria (Kawada et al., 2011c). These regions are one of the focal points identified as a high vector transmission region in Kenya, and more than 50% of the population is exposed to ≥40% $PfPR_{2\text{-}10}$ (*Plasmodium falciparum* parasite rate corrected to a standard age-range of 2 to less than 10 years old) (Noor et al., 2009) and accordingly high coverage of LLINs or ITNs has been accomplished. In fact, the percentages of households that have at least one LLIN in Nyanza and Western provinces (>70%) were fairly higher than the other provinces (<70%) (Kenya HDS Final Report, 2009). Moreover, high population density of *An. gambiae* s.s. and *An. arabiensis* in the above regions (Okara et al., 2010), as well as high human population density, might have increased the contact frequency of vector mosquitoes to LLITN/ITN, resulting in the high selection pressure with pyrethroids. Mathias et al. (2011) reported that the East African *kdr* allele (L1014S) coincidentally increased in frequency during the past decade in *An. gambiae* s.s. in western Kenya, most of which are homozygous *kdr* allele, as household ownership of insecticide-treated bed nets increased regionally.

Several factors are believed to play major roles in inducing pyrethroid resistance in mosquitoes. The most serious factor is the uncontrolled use of photo-stable pyrethroids. Photo-stable pyrethroids persist on substrates such as wall and floor surfaces for long periods and hence continue to kill insects that make contact with these substrates. This induces a strong selection pressure on the insect population resulting in a population of resistant offspring. In the past, the use of pyrethroids in aqueous environments was impossible since pyrethroids are highly toxic to aqueous organisms. However, recently, a new pyrethroid with low fish toxicity has been commercially produced and is widely used in aqueous environments such as paddy fields. This might cause considerable selection pressure on the mosquito larvae distributed in such environments. The most serious problem is that resistance to a single pyrethroid causes cross-resistance to all other pyrethroids, including knockdown agents. In fact, many reports concerning pyrethroid resistance have emerged after the successful application of pyrethroids as vector control agents. Therefore, the uncontrolled use of such pyrethroids might lead to the end of the golden age of pyrethroids.

Humans have invented insecticides to ensure comfort and to achieve ideal conditions. Good insecticides, therefore, should be as effective as possible so that the above mentioned goals are realized. However, the development and manufacturing costs of insecticides should be as low as possible (Kawada, 2009c). It is, therefore, our duty to use insecticides in the most effective and prudent manner possible in order to maintain their effectiveness and sustain their use. In order to effectively manage pyrethroid resistance, the establishment of a feasible insecticide management system and a regular monitoring system of pyrethroid susceptibility will be essential. Moreover, it is expected that new self-protection measures using exito-repellent type I pyrethroids are of great interest as substitutional or supplemental techniques for bio-rational vector control measures in the future, as well as reconsideration of the use of photo-unstable knockdown agents as spatial repellents, which effectively interfere with disease transmission without causing any selection pressure to insect populations.

6. Acknowledgment

We thank all staffs in Thomas Odhiambo campus of the International Center of Insect Physiology and Ecology (ICIPE), Mbita, Kenya; Kenya Medical Research Institute, Nairobi, Kenya; National Institute of Hygiene and Epidemiology, Hanoi, Vietnam; Pasteur Institute of Ho Chi Minh, Vietnam; National Institute of Infectious Diseases, Tokyo, Japan; Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan, and Sumitomo Chemical Co., Ltd., Hyogo, Japan, for providing technical support and for assisting with this study.

7. References

Arugueta, T. B. O.; Kawada, H.; Sugano, M.; Kubota, S.; Shono, Y.; Tsushima, K. & Takagi, M. (2004). Comparative insecticidal efficacy of a new pyrethroid, metofluthrin, against colonies of Asian *Culex quinquefasciatus* and *Culex pipiens pallens*. *Medical Entomology and Zoology*, Vol.55, pp. 289-294

Binka, F. N.; Kubaje, A.; Adjuik, M.; Williams, L. A.; Lengeler, C.; Maude, G. H.; Arma, G. E.; Kajihra, B.; Adiama, J. H.; Lengeler, C. & Smith, P. G. (1996). Impact of permethrin treated bed nets in child mortality in Kassena-Nankana district, Ghana: a

randomized controlled trial. *Tropical Medicine & International Health*, Vol.1, pp. 147-154

Birley, M. H.; Mutero, C. M.; Turner, I. F. & Chadwick, P. R. (1987). The effectiveness of mosquito coils containing esbiothrin under laboratory and field conditions. *Annals of Tropical Medicine and Parasitology*, Vol.81, pp. 163-171

Cornel, A. J.; Stanich, M. A.; McAbee, R. D. & Mulligan III, F. S. (2002). High level methoprene resistance in the mosquito *Ochlerotatus nigromaculis* (Ludlow) in Central California. *Pest Managment Science*, Vol.58, pp. 791-798

Curtis, C. F.; Myamba, J. & Wilkes, T. J. (1996). Comparison of different insecticides and fabrics for anti-mosquito bednets and curtains. *Medical and Veterinary Entomology*, Vol.10, pp. 1-11

Dabire, R. K.; Diabate, A.; Baldet, T.; Paré-Toé, L.; Guiguemde, R. T.; Ouédraogo, J. B. & Skovmand, O. (2006) Personal protection of long lasting insecticide-treated nets in areas of *Anopheles gambiae* s.s. resistance to pyrethroids. *Malaria Journal*, Vol.5, pp. 12

Das, M. L.; Roy, L.; Rijal, S.; Paudel, I. S.; Picado, A.; Kroeger, A.; Petzold, M.; Davies, C. & Boelaert, M. (2010). Comparative study of kala-azar vector control measures in eastern Nepal. *Acta Tropica*, Vol.113, pp. 162-166

Devine, G. J.; Perea E. Z.; Killeen, G. F.; Stancil, J. D.; Clark, S. J. & Morrison, A. C. (2009). Using adult mosquitoes to transfer insecticides to *Aedes aegypti* larval habitats. *Proceedingsof the National Academy of Sciences of the United States of America*, Vol.106. pp. 11530-11534

Dinesh, D. S.; Das, P.; Picado, A.; Davies, C.; Speybroeck, N.; Ostyn, B.; Boelaert, M. & Coosemans, M. (2008). Long-lasting insecticidal nets fail at household level to reduce abundance of sandfly vector *Phlebotomus argentipes* in treated houses in Bihar (India). *Tropical Medicine & International Health*, Vol.13, pp. 953-958

Emami, M. M.; Yazdi, M. & Guillet, P. (2009). Efficacy of Olyset long-lasting bednets to control transmission of cutaneous leishmaniasis in Iran. *East Mediterranean Health Journal*, Vol.15, pp. 1075-1083

Faiman, R.; Cuño, R. & Warburg, A. (2009). Control of phlebotomine sand flies with vertical fine-mesh nets. *Journal of Medical Entomology*, Vol.46, pp. 820-831

Guillet, P.; Alnwick, D.; Cham, M. K.; Neira, M.; Zaim, M.; Heymann, D. & Mukelabai, K. (2001). Long-lasting treated mosquito nets: a breakthrough in malaria prevention. *Bulletin of World Health Organization*, Vol.79, pp. 998

Henrick, C. A.; Staal, G. B. & Siddall, J. B. (1973). Alkyl 3,7,11 trimethyl 2,4 dodecadienoates, a new class of potent insect growth regulators with juvenile hormone activity. *Journal of Agricultural and Food Chemistry*, Vol.21, pp. 354-359.

Hirano, M.; Hatakoshi, M.; Kawada, H. & Takimoto, Y. (1998). Pyriproxyfen and other juvenile hormone analogues. *Review in Toxicology*, Vol.2, pp. 357-394

Igarashi, A. (1997). Impact of dengue virus infection and its control. *FEMS Immunology and Medical Microbiology*, Vol.18, pp. 291-300

Ishaaya, I.; Kontsedalov, S. & Horowitz, A. R. (2005). Biorational insecticides: Mechanism and Cross-resistance. *Archives of Insect Biochemistry and Physiology*, Vol.58, pp. 192-199

Itoh, T. (1993). Control of DF/DHF vector, *Aedes* mosquito, with insecticides. *Tropical Medicine*, Vol.35, pp. 259-267

Itoh, T.; Kawada, H.; Abe, Y.; Eshita, Y.; Rongsriyam, Y. & Igarashi, A. (1994). Utilization of blood-fed females of *Aedes aegypti* as a vehicle for the transfer of the insect growth regulator pyriproxyfen to larval habitats. *Journal of American Mosquito Control Association*, Vol.10, pp. 344-347

Iwashita, H.; Dida, G.; Futami, K.; Sonye, G.; Kaneko, S.; Horio, M.; Kawada, H.; Maekawa, Y.; Aoki, Y. & Minakawa, N. (2010). Sleeping arrangement and house structure affect bed net use in villages along Lake Victoria. *Malaria Journal*, Vol.9, pp. 176

Iwanaga, K. & Kanda, T. (1988). The effects of a juvenile hormone active oxime ether compound on the metamorphosis and reproduction of an Anopheline vector, *Anopheles balabacensis*. *Applled Entomology and Zoology*, Vol.23, pp. 186-193

Jeyalakshmi, T.; Shanmugasundaram, R. & Murthy, P. B. (2006). Comparative efficacy and persistency of permethrin in Olyset net and conventionally treated net against *Aedes aegypti* and *Anopheles stephensi*. *Journal of American Mosquito Control Association*, Vol.22, pp. 107-110

Judson, C. L. & Lumen, H. Z. (1976). Some effects of juvenile hormone and analogs on ovarian follicles of the mosquito *Aedes aegypti* (Diptera: Culicidae). *Journal of Medical Entomology*, Vol.13, pp. 197-201

Kawada, H.; Itoh, T.; Abe, Y. & Horio, M. (1993). Can mosquito be a carrier of larvicides ? *In*: Proceedings of the 1st International Congress on Insect Pests in the Urban Environment, Cambridge, UK, pp. 497

Kawada, H.; Maekawa, Y.; Tsuda, Y. & Takagi, M. (2004a). Laboratory and field evaluation of spatial repellency with metofluthrin-impregnated paper strip against mosquitoes in Lombok Island, Indonesia. *Journal of American Mosquito Control Association*, Vol.20, pp. 292-298

Kawada, H.; Maekawa, Y.; Tsuda, Y. & Takagi, M. (2004b). Trial of spatial repellency of metofluthrin-impregnated paper strip against *Anopheles* and *Culex* in shelters without walls in Lombok, Indonesia. *Journal of American Mosquito Control Association*, Vol.20, pp. 434-437

Kawada, H.; Maekawa, Y. & Takagi, M. (2005a). Field trial of the spatial repellency of metofluthrin-impregnated plastic strip against mosquitoes in shelters without walls (Beruga) in Lombok, Indonesia. *Journal of Vector Ecology*, Vol.30, pp. 181-185

Kawada, H.; Yen, Ng. T.; Hoa, Ng. T.; Sang, Tr. M.; Dan, Ng. V. & Takagi, M. (2005b). Field evaluation of spatial repellency of metofluthrin impregnated plastic strips against mosquitoes in Hai Phong city, Vietnam. *American Journal of Tropical Medicine and Hygien*, Vol.73, pp. 350-353

Kawada, H.; Iwasaki, T.; Loan, L. L.; Tien, T. K.; Mai, N. T.N.; Shono, Y.; Katayama, Y. & Takagi, M. (2006a). Field evaluation of spatial repellency of metofluthrin-impregnated latticework plastic strips against *Aedes aegypti* (L.) and analysis of environmental factors affecting its efficacy in My Tho city, Tien Giang, Vietnam. *American Journal of Tropical Medicine and Hygien*, Vol.75, pp. 1153-1157

Kawada, H., Saita, S.; Shimabukuro, K.; Hirano, M.; Koga, M.; Iwashita, T. & Takagi, M. (2006b). Effectiveness in controlling mosquitoes with EcoBio-Block® S - a novel integrated water purifying concrete block formulation combined with the insect

growth regulator pyriproxyfen. *Journal of American Mosquito Control Association*, Vol.22, pp. 451-456

Kawada, H.; Temu, E. A.; Minjas, J. N.; Matsumoto, O.; Iwasaki, T. & Takagi, M. (2008). Field evaluation of spatial repellency of metofluthrin-impregnated plastic strips against Anopheles gambiae complex in Bagamoyo, coastal Tanzania. *Journal of American Mosquito Control Association*, Vol.24, pp. 404-409

Kawada, H.; Higa, Y.; Nguyen, T. Y.; Tran, H. S.; Nguyen, T. H. & Takagi, M. (2009a). Nationwide investigation on the pyrethroid-susceptibility of mosquito larvae collected from used tires in Vietnam. *PLoS Neglected Tropical Diseases*, Vol.3, e0000391

Kawada, H.; Higa, Y.; Komagata, O.; Kasai, S.; Tomita, T.; Nguyen, T. H.; Luu, L. L.; Sánchez, R. A. P. & Takagi, M. (2009b). Widespread distribution of a newly found point mutation in voltage-gated sodium channel in pyrethroid-resistant *Aedes aegypti* populations in Vietnam. *PLoS Neglected Tropical Diseases*, Vol.3, e0000527

Kawada, H. (2009c). An Inconvenient Truth of Pyrethroid - Does it have a promising future? -. *In*: J. Clark, J. R. Bloomquist, H. Kawada [ed.] Advances in Human Vector Control (ACS Symposium Book 1014) American Chemical Society, New York.

Kawada, H.; Dida, G. O.; Ohashi, K.; Komagata, O.; Kasai, S.; Tomita, T.; Sonye, G.; Maekawa, Y.; Mwatele, C.; Njenga, S. M.; Mwandawiro, C.; Minakawa, N. & Takagi, M. (2011a). Multimodal pyrethroid resistance in malaria vectors *Anopheles gambiae* s.s., *Anopheles arabiensis*, and *Anopheles funestus* s.s. in western Kenya. *PLoS ONE,* Vol. 6, e22574

Kawada, H.; Dida, G. O.; Ohashi, K.; Sonye, G.; Maekawa, Y.; Njenga, S. M.; Mwandawiro, C.; Minakawa, N. & Takagi, M. (2011b). An evaluation of the insecticide-impregnated ceiling nets with coarse mesh size as a barrier against the invasion of malaria vectors. *PLoS ONE, in review*

Kawada, H.; Futami, K.; Komagata, O.; Kasai, S.; Tomita, T.; Sonye, G.; Mwatele, C.; Njenga, S. M.; Mwandawiro, C.; Minakawa, N. & Takagi, M. (2011c). Distribution of a knockdown resistance mutation (L1014S) in *Anopheles gambiae* s.s. and *Anopheles arabiensis* in Western and Southern Kenya. *PLoS ONE,* Vol. 6, e24323

Kasili, S.; Kutima, H.; Mwandawiro, C.; Ngumbi, P. M.; Anjili, C. O. & Enayati, A. A. (2010). Laboratory and semi-field evaluation of long-lasting insecticidal nets against leishmaniasis vector, *Phlebotomus* (*Phlebotomus*) *duboscqi* in Kenya. *Journal of Vector Borne Diseases*, Vol.47, pp. 1-10

Kirby, M. J.; Ameh, D.; Bottomley, C.; Green, C.; Jawara, M.; Milligan, P. J.; Snell, P. C.; Conway, D. J. & Lindsay, S. W. (2009). Effect of two different house screening interventions on exposure to malaria vectors and on anaemia in children in The Gambia: a randomised controlled trial. *Lancet*, Vol.374(9694), pp. 998-1009

Klowden, M. J. & Chambers, G. M. (1989). Ovarian development and adult mortality in *Aedes aegypti* treated with sucrose, juvenile hormone and methoprene. *Journal of Insect Physiology*, Vol.35, pp. 513-517

Kroeger, A.; Lenhart, A.; Ochoa, M.; Villegas, E.; Levy, M.; Alexander, N. & McCall, P. J. (2006). Effective control of dengue vectors with curtains and water container covers treated with insecticide in Mexico and Venezuela: cluster randomised trials. *British Medical Journal*, Vol.332(7552), pp. 1247-1252

Lengeler, C.; Cattani, J. & De Savigny, D. (1996). Net gain. A new method for preventing malaria deaths. IDRC Otawa/WHO, Geneva, pp. 189

Lindsay, S. W.; Jawara, M.; Paine, K.; Pinder, M.; Walraven, G. E. L. & Emerson, P. M. (2003). Changes in house design reduce exposure to malaria mosquitoes. *Tropical Medicine & International Health*, Vol.8, pp. 512-517

Lines, J. (1996). Mosquito nets and insecticides for nets treatment: a discussion of existing and potential distribution systems in Africa. *Tropical Medicine & International Health*, Vol.1, pp. 616-632

MacIver, D. R. (1964). Mosquito coils Part II. Studies on the action of mosquito coil smoke on mosquitoes. *Pyrethrum Post*, Vol.7, pp. 7-14

Mathias, D. K.; Ochomo, E.; Atieli, F.; Ombok, M.; Bayoh, M. N.; Olang, G.; Muhia, D.; Kamau, L.; Vulule, J. M.; Hamel, M. J.; Hawley, W. A.; Walker, E. D. & Gimnig, J. E. (2011). Spatial and temporal variation in the *kdr* allele L1014S in *Anopheles gambiae* s.s. and phenotypic variability in susceptibility to insecticides in Western Kenya. *Malaria Journal*, Vol.14, pp. 10

Miyamoto, J.; Hirano, M.; Takimoto, Y. & Hatakoshi, M. (1993). Insect growth regulators for pest control, with emphasis on juvenile hormone analogs. Present status and future prospects, In: *Pest control with enhanced environmental safety (ACS symposium series 524)*, Duke, S. O.; Menn, J. J. & Plimmer, J. R., (Ed.), 144-168, American chemical society, Washington DC

Nam N. V.; de Vries, P. J.; Toi, L. V. & Nagelkerke, N. (2005) Malaria control in Vietnam: the Binh Thuan experience. Tropical Medicine & International Health, Vol.10, pp. 357-365

Nam, V. S.; Yen, N. T.; Holynska, M.; Reid, J. W. & Kay, B. H. (2000). National progress in dengue vector control in Vietnam: survey for Mesocyclops (Copepoda), Micronecta (Corixidae), and fish as biological control agents. *American Journal of Tropical Medicine and Hygien*, Vol.62, pp. 5-10

Nevill, C. G.; Some, E. S.; Mung'ala, V. O. & Mutemi, W.; New, L.; Marsh, K.; Lengeler, C. & Snow, R. W. (1996). Insecticide-treated bed nets reduce mortality and severe morbidity from among children on the Kenyan Coast. *Tropical Medicine & International Health*, Vol.1, pp. 139-146

Njie, M.; Dilger, E.; Lindsay, S. W. & Kirby, M. J. (2009). Importance of eaves to house entry by anopheline, but not culicine, mosquitoes. *Journal of Medical Entomology*, Vol.46, pp. 505-510

Noor, A. M.; Gething, P. W.; Alegana1, V. A.; Patil, A. P.; Hay, S. I.; Muchiri, E.; Juma, E. & Snow, R. W. (2009). The risks of malaria infection in Kenya in 2009. *BMC Infectios Diseases*, Vol.9, pp. 180

Ohba, S.; Ohashi, K.; Pujiyati, E.; Higa, Y.; Kawada, H. & Takagi, M. (2011). The effect of juvenile hormone analogue, pyriproxyfen, as a "population growth regulator" against *Aedes albopictus* in semi-field conditions. *Unpublished*

Okara, R. M.; Sinka, M. E.; Minakawa, N.; Mbogo, C. M.; Hay, S. I. & Snow, R. W. (2010). Distribution of the main malaria vectors in Kenya. *Malaria Journal*, Vol.9, pp. 69

Pålsson, K.; Jaenson, T. G. T.; Dias, F.; Laugen, A. T. & Björkman, A. (2004). Endophilic *Anopheles* mosquitoes in Guinea Bissau, West Africa, in relation to human housing conditions. *Journal of Medical Entomology*, Vol.41, pp. 746-752

Phong, T. V. & Nam, V. S. (1999). Key breeding sites of dengue vectors in Hanoi, Vietnam, 1994-1997. *Dengue Bulletin*, Vol.23, pp. 67-72

Schechter, M. S.; Green, N. & Laforge F. B. (1949). Constituents of pyrethrum flowers XXIII. Cynerolone and the synthesis of related cyclopentenolones. *Journal of American Chemical Society*, Vol.71, pp. 3165-3173

Siegert, P. Y.; Walker, E. & Miller, J. R. (2009). Differential behavioral responses of *Anopheles gambiae* (Diptera: Culicidae) modulate mortality caused by pyrethroid-treated bednets. *Journal of Economic Entomology*, Vol.102, pp. 2061-2071

Sihuincha, M.; Zamora-Perea, E.; Orellana-Rios, W.; Stancil, J. D.; López-Sifuentes, V.; Vidal-Oré, C. & Devine, G. J. (2005). Potential use of pyriproxyfen for control of *Aedes aegypti* (Diptera: Culicidae) in Iquitos, Perú. *Journal of Medical Entomology*, Vol.42, pp. 620-630

Snow, W. F. (1987). Studies of house-entering habits of mosquitoes in The Gambia, West Africa: experiments with prefabricated huts with varied wall apertures. *Medical and Veterinary Entomology*, Vol.1, pp. 9-21

Syafruddin; Arakawa, R.; Kamimura, K. & Kawamoto, F. (1990). Histopathological effects of an insect growth regulator, 4-phenoxyphenyl (*RS*)-2-(2-pyridyloxy)propyl ether (pyriproxyfen), on the larvae of *Aedes aegypti*. *Japanese Journal of Sanitary Zoology*, Vol.41, pp. 15-22

Trayler, K. M. & Davis, J. A. (1996). Sensitivity of *Daphnia carinata* sensu lato to the insect growth regulator, pyriproxyfen. *Ecotoxicolgy and Environmental Safety*, Vol.33, pp. 154-156

Tsunoda, T.; Kawada, H.; Trang, H. T. T.; Loan, L. L.; San, L. H.; Huu, T. N.; Huong, V. T. Q.; Hasebe, F.; Tsuzuki, A. & Takagi, M. (2011). Effect of controlled release plastic formulations of permethrin (Olyset® Net) as water container lid and controlled release formulation of an insect growth regulator, pyriproxyfen, on field populations of *Aedes aegypti* (L.) in southern Vietnam. *PLoS Neglected Tropical Diseases, in review*

Tsuzuki, A.; Huynh, T.; Tsunoda, T.; Luu L. L.; Kawada, H. & Takagi, M. (2009). Effect of existing practices on reducing *Aedes aegypti* pre-adults in key breeding containers in Ho Chi Minh City, Vietnam. *American Journal of Tropical Medicine and Hygien*, Vol.80, pp. 752-757

Ujihara, K.; Mori, T.; Iwasaki, T.; Sugano, M.; Shono, Y. & Matsuo, N. (2004). Metofluthrin: A potent new synthetic pyrethroid with high vapor activity against mosquitoes. *Bioscience, Biotechnology, and Biochemistry*, Vol.68, pp. 170-174

Vanlerberghe, V.; Villegas, E.; Oviedo, M.; Baly, A.; Lenhart, A.; McCall, P. J. & Van der Stuyft, P. (2011a). Evaluation of the effectiveness of insecticide treated materials for household level dengue vector control. *PLoS Neglected Tropical Diseases*, Vol.5, e994

Vanlerberghe, V.; Villegas, E.; Jirarojwatana, S.; Santana, N.; Trongtorkit, Y.; Jirarojwatana, R.; Srisupap, W.; Lefèvre, P. & Van der Stuyft, P. (2011b). Determinants of uptake, short-term and continued use of insecticide-treated curtains and jar covers for dengue control. *Tropical Medicine & International Health*, Vol.16, pp. 162-173

Vu, D. H.; Nguyen, T. B. N.; Do, T. H. & Nguyen, T. B. L. (2004). Susceptibility of *Aedes aegypti* to insecticides in Viet Nam. *Dengue Bulletin*, Vol.28, pp. 179-183

Williams, C. M. (1967). Third-generation pesticides. *Scientific American*, Vol.217, pp. 13-17

Winney, R. (1975). Pyrethrins and pyrethroids in coils–a review. *Pyrethrum Post*, Vol.13, pp. 17-22

Zaim, M. & Jambulingam, P. (2007). Global insecticide use for vector-borne disease control, World Health Organization

Zhang, L.;, Kasai, S. & Shono, T. (1998). In vitro metabolism of pyriproxyfen by microsomes from susceptible and resistant housefly larvae. *Archives of Insect Biochemistry and Physiology*, Vol.37, pp. 215-224

30

Insecticides for Vector-Borne Diseases: Current Use, Benefits, Hazard and Resistance

Yousif E. Himeidan[1], Emmanuel A. Temu[2] and Eliningaya J. Kweka[3]
[1]Entomology Unit, Faculty of Agriculture and Natural Resources, University of Kassala, New Halfa,
[2]Department of Epidemiology & Population Health, London School of Hygiene & Tropical Medicine London,
[3]Tropical Pesticides Research Institute, Division of Livestock and Human Diseases Vector Control, Arusha,
[1]Sudan
[2]United Kingdom
[3]Tanzania

1. Introduction

"Insect vector-borne disease" is the term commonly used to describe an illness/or disease caused by an infectious microbe that is transmitted to human by blood-sucking arthropods such as mosquitoes (e.g. malaria, dengue fever, yellow fever, encephalitis, filariasis, West Nile fever and chikungunya), ticks (e.g. Lyme disease), sandflies (e.g. leishmaniasis), tsetse fly (e.g. African trypanosomiasis) and kissing bug (e.g. Chagas disease). These diseases are a global problem, represent a significant threat to human health and cause enormous impact on economic and social life despite considerable national and international control efforts, i.e. malaria alone kills annually around one million peoples (Figure 1 & Table 1) (WHO, 2004; 2010a). It has been well documented by the World Health Organisation (WHO) and in numerous scientific investigations and reports that the use of synthetic insecticides can dramatically reduce the risk of insect-vector-borne diseases, particularly in the case of malaria (Hemingway and Bates, 2003; WHO, 2006a). Current vector control strategies rely heavily on use of insecticides through insecticide-treated nets (ITNs) and indoor residual spraying (IRS) for example. Space spraying constitutes the first line of activity in case of epidemics. Larval control by using insecticides was a success in the past in eradicating malaria in some parts of the world i.e. the Anopheles gambiae Project in Egypt (Shousha,1948) but still do not received much interest in the current strategies.

The current success of IRS and ITNs in reducing malaria, the most deadly vector-borne disease, contributed towards the optimism that elimination of this disease as a public health problem is a feasible objective (Roberts and Enserink, 2007). Substantial international efforts have been made during the last three years enabling distribution to approximately 289 million ITNs in sub-Saharan Africa, enough to cover 76% of the 765 million people at risk of malaria (WHO, 2010). The number of countries that employed IRS as vector control strategy increased from 31 in 2007 to 68 in 2009 (WHO, 2010). Further scale up of IRS and ITNs for

malaria prevention and vector control is occurring throughout the African continent. However, the huge amount of vector control insecticides is used for IRS which represents around 90% of the total quantity of the annual global quantity of insecticide utilized for vector control (WHO, 2010b; Zaim, 2002; Zaim and Jambulingam, 2004; 2007).

Disease	Vector	Disease burden DALYs[1] (thousands) [2]	Deaths (thousands) [2]
Malaria	Anopheles mosquitoes	32 342	838
African Trypanosomiasis	Tsetse flies	1 409	44
Leishmaniasis	Sandflies	1 486	36
Japanese encephalitis	Culex mosquitoes	790	14
Dengue	Aedes mosquitoes	470	13
Chagas disease	Triatomid bugs	342	10
Lymphatic filariasis	Anopheles and Culex mosquitoes	4 879	0
Onchocerciasis	Blackflies	348	0

[1]DALYs, disability adjusted life years.
[2]Adapted from World Health Organization (WHO), projections of mortality and burden of disease, 2004-2030, baseline scenario 2008 (see: http://www.who.int/healthinfo/global_burden_disease/projections/en/index.html).

Table 1. The current burden of vector-borne diseases.

Several insecticides have historically been used for IRS, the first and most well-known being Dichloro Diphenyl Trichloroethane (DDT). According to the World Health Organization position statement (WHO, 2011), DDT is still needed for vector control simply because in some places there is no alternative of equivalent efficacy and operational feasibility. To date, no change has been warranted in the existing WHO recommendations on the use of DDT for IRS. However, the possible adverse consequences of human exposure to DDT cannot be ignored, even with limited evidence, and merit further revision. Yet, pyrethroids (PYs) are the most commonly insecticides used for IRS and also are the only compounds currently approved by the WHO Pesticide Evaluation Scheme (WHOPES) for ITNs (WHO, 2007). Even limited risk assessments undertaken regard to the safety of personal use of ITNs suggested a high margin of safety for PYs (Bomann, 1995; Zaim et al., 2000), we do not know the real consequences of large scale use of PYs on the environment and human health. Indeed in understanding results of these limited risk assessments, it is important to note that even the use of mosquito nets is not new, long term use of long-lasting insecticide-treated bed nets (LLINs), the new generation of ITNs, and in a large scale community-based intervention is a new technology, and some uncertainty remains about the potential for health problems i.e. the potential chronic neuro-behavioural toxicity in humans (Kolaczinski and Curtis, 2004).

Realizing a scaling up of the current vector control methodologies could lead to deploy of tens of millions of doses of insecticides in the form of ITNs and IRS over millions of homes in endemic countries annually. Thus, strategies to ensure a fuller understanding of potential health risks induced by massive use of insecticides and to minimize actual and potential adverse effects on human health are urgently needed. The risks to public health by deployment of DDT or other insecticides must be carefully weighed against the benefits, in

this case the prevention of vector-borne diseases. Moreover, there are strong evidences that more insect vectors species are becoming resistant to the toxic action of these insecticides and through different resistance mechanisms, especially knock-down resistance (kdr) mechanism to DDT and pyrethroids (Rivero et al., 2010; Ranson et al., 2011). The spread of insecticide resistance vertically to new species and horizontally to new countries poses a great danger likely to undermine the contribution of vector control efforts to control of diseases. Based on screening scientific evidences from literature review, discussion in this chapter, will be focused on current status, benefits, resistance and potential hazardous effects on human health of insecticide vectors management.

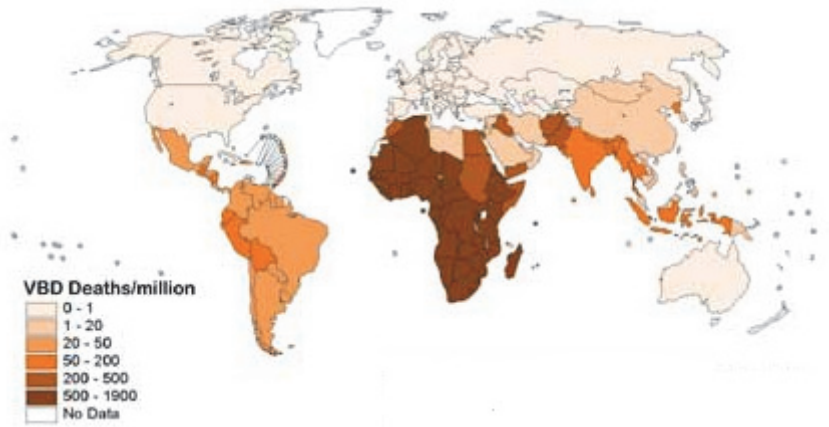


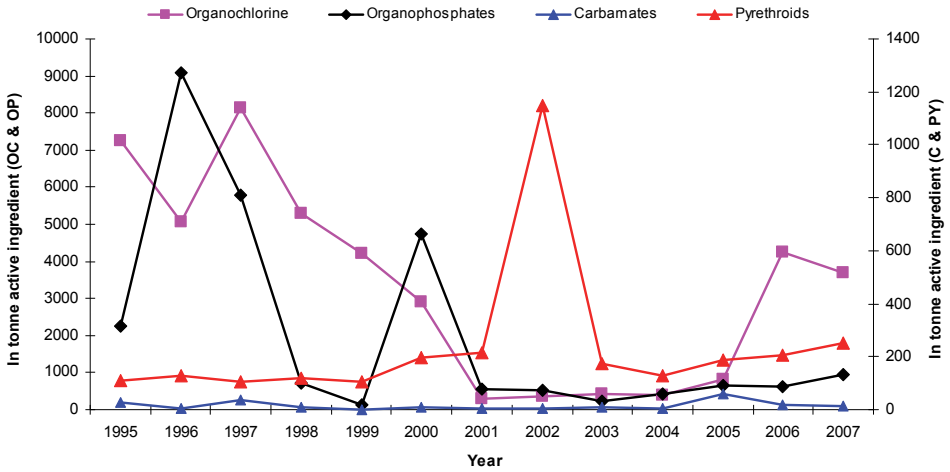
Fig. 1. Death from Vector-Born Diseases (VBD). Source: World Health Organization, Health and Environment Linkages Initiative, available at:
http://www.who.int/heli/risks/vectors/vector/en/index.html

2. Current status of global insecticides use for insect vectors control

On average, about 3962 metric tonnes of active ingredient of organochlorines, 795 tonnes of organophosphates, 16 tonnes of carbamates and 229 tonnes of pyrethroids were reportedly used annually for vector control at the global level during 2006–2007. Compared to previous years of 2000 – 2002, the recent global insecticide use for vector control increased by 333.5% for organochlorines (DDT) and 224% for carbamates. The trend in the global use of insecticides for vector control during 1995 – 2007 is shown in Figure 2.

Compare to 1990s, there is a global decline trend in DDT use, but still on an average, 40,000 tonnes of DDT were used annually during 2006 - 2007 for vector control (Figure 2).This is similar to the annual amount used during the malaria eradication period of 1955–1970. Concerns about the continued use of DDT are fuelled by recent reports of high levels of human exposure associated with IRS amid accumulating scientific evidence on chronic health effects (Sadasivaiah et al., 2007). However, there was a reduction in the use of pyrethroids and organophosphates insecticides. Only 44% and 41% of the total amount of pyrethroids and organophosphates used in 2000-2002 were applied during 2006-2007, respectively (Table 2). Overall, there was a great reduction in use of active ingredient of organochlorines, organophosphates, and carbamates during 2000s compared with 1990s. In

contrast, the use of pyrethroids increased sharply during 2001-2003, corresponding to the period of scaling up of the old generation of ITNs, which required re-treatment by pyrethroids insecticide every six months.



[1] (Data source: Zaim, 2002; Zaim and Jambulingam, 2004; 2006; Ameneshewa et al., 2009).

Fig. 2. Trend in the global use of insecticides for vector control reported to WHOPES, by class of insecticide, 1995–2007.[1]

During 2006–2007, about 90% of the total quantity of all classes of insecticides was reportedly used for IRS for vector control, followed by space spraying (4%), larviciding (3.8%), treatment of mosquito nets (0.3%) and other applications (0.6%).

Year	Insecticide Class[2]	WHO region						
		African	Americas	Eastern Mediterranean	European	South-East Asia	Western Pacific	All regions
2000-2002	OC	307 445	725	0	0	879 761	0	1 187 931
	OP	34 170	358 136	58 988	6 660	1 482 000	6 205	1 946 158
	C	331	2 811	1 481	133	1 237	0	6 993
	PY	2 688	460 940	7 901	1 278	17 257	29 071	519 134
2003-2005	OC	546 909	0	0	0	0	0	546 909
	OP	11 707	367 827	21 148	3 317	24 554	8 686	437 239
	C	20 307	2 681	622	0	1 920	0	24 230
	PY	13 606	91 948	15 375	1 411	9 979	29 553	161 872
2006-2007	OC	755 179	0	0	0	3 206 931	9	3 962 118
	OP	6 403	466 233	52 398	1 620	226 951	41 265	794 868
	C	6 137	781	7 148	1 076	520	0	15 662
	PY	6 616	108 450	26 802	3 228	28 927	54 891	228 913

[1] (Data source: Zaim, 2002; Zaim and Jambulingam, 2004; 2006; Ameneshewa et al., 2009).
[2] OC= Organochlorines exclusively DDT; OP= Organophosphates; C= Carbamates; PY= Pyrethroids.

Table 2. Global use of insecticides for vector control reported to WHOPES, in kg of active ingredient, by class of insecticides and WHO region, 2000–2007.[1]

While the same order of application methods was also reported for 2003–2005, there was a marked increase in the proportion of insecticides used for IRS (from 60%) and a marked decrease in the proportion of insecticides used for space spraying (from 30.7%). There was also reduction in the annual insecticide used for larviciding in 2007 compared with 2000 indicating decrease of interest in relaying on use of these methods for vectors controls (Table 3).

Type of application	Insecticide Class	Amount of insecticide used (kg active ingredient)							
		2000	2001	2002	2003	2004	2005	2006	2007
IRS	OC	2 921 050	291 800	350 941	423 868	389 210	827 648	4 232 505	3 691 730
	OP	4 263 167	184 782	70925	48 312	32 070	91 232	323 706	583 548
	C	9 472	4 007	5 277	6 242	4 417	61 235	17 798	11 761
	PY	96 413	96 402	659 458	82 164	71 183	55 128	122 957	121 912
ITN	PY	43 650	18 253	9 426	15 838	4 694	31 819	6 989	21 412
Larviciding	OP	245 556	112 871	123 659	90 725	89 426	83 136	187 935	189 518
Space spraying	OP	238 929	246 271	324 741	84 243	311 948	473 994	106 643	173 736
	PY	51 090	57 718	56 604	49 675	50 336	98 021	41 233	94 219
Other applications	OP	N.A[2]	N.A	N.A	N.A	N.A	N.A	12 682	10 717
	C	N.A	N.A	N.A	N.A	N.A	N.A	1 019	746
	PY	N.A	N.A	N.A	N.A	N.A	N.A	22 182	10 131

[1] (Data source: Zaim and Jambulingam, 2004; 2006; Ameneshewa et al., 2009).
[2]N.A= Data is not available

Table 3. Recent use of insecticides for vector control reported to WHOPES, in kg of active ingredient, by type of application and class of insecticide, 2000–2007.[1]

The use of organochlorines at global level was reportedly limited only to IRS and the increased reported during 2007 was 126.4% compared with 2000. Except for a report from India on the use of hexachlorocyclohexane (HCH) in 2000, DDT has been the only organochlorines insecticide reportedly used at global level annually for vector control (Zaim and Jambulingam, 2004). This is in agreement with the current approval by WHOPES of insecticides recommended for IRS (Table 4). The organochlorines insecticide had been intensively applied during 1990s and the amount used during this period was more than 5000 metric tonnes of DDT active ingredient, and then decreased to its minimum level (500 metric tonnes) during 2003-2005. Now vectors control activities relayed heavily on the use of pyrethroids insecticides for ITNs and IRS. The use of organochlorines raised slightly again after reintroduction of DDT in 2005 for malaria vector control in several countries of Africa.
Carbamates were mainly used for IRS (94%) and in smaller quantity for other applications (6%) such as dusting, painting and peri-focal treatment. Of the total annual use of organophosphates during 2006-2007, 57% was used for indoor residual spraying, 23.8% for larviciding, 17.7% for space spraying and 1.5% for other applications. While about 55.5% of the total use of pyrethroids was for indoor residual spraying and 30.7% for space spraying, 6.4% was used for treatment of mosquito nets and 7.3% for other applications. In general, the use of

pyrethroids for IRS was increased by 126.5% in 2007 compared with 2000. Interestingly, despite the numerous scaling up and the high coverage achieved, there was a reduction of almost 50% in the annual use of pyrethroids insecticides for treatment of nets in 2007 compared with 2000. This is due to the starting use of the new generation of net, the long lasting insecticidal nets (LLINs) in 2006. LLINs are nets treated in the factory with an insecticide incorporated into the net fabric which makes the insecticide last at least 20 washes in standard laboratory testing and three years of recommended use under field conditions. LLINs are being promoted by WHO and Roll Back Malaria partners as a cost effective and sustainable method for protection against malaria. With LLINs therefore the enormous amount of insecticides requested for retreating old nets is no longer needed.

2.1 Insecticides recommended for IRS

Indoor residual spraying (IRS) is a major intervention for malaria control (WHO, 2006b). There are currently 12 insecticides recommended for IRS, this includes 1 organochlorine, 3 organophosphates, 2 carbamates and 6 pyrethroids insecticides. The only insecticide approved for vector control from organochlorines is DDT. Dosage, toxicity, WHO hazard classification and registration status of DDT and other insecticides recommended for IRS, at U.S Environmental Protection Agency (EPA) are shown in Table 4.

Insecticide compounds and formulations[2]	Class group[3]	Dosage (g a.i./m²)	Oral toxicity for rats (LD50 of a.i. mg/kg)	Duration of effective action (months)	WHO Class[4]	EPA Status[5]
DDT WP	OC	1-2	113	>6	II	Cancelled
Malathion WP	OP	2	2100	2-3	III	Active
Fenitrothion WP	OP	2	503	3-6	II	Active
Pirimiphos-methyl WP & EC	OP	1-2	2018	2-3	III	Active
Bendiocarb WP	C	0.1-0.4	55	2-6	II	Cancelled
Propoxur WP	C	1-2	95	3-6	II	Active
Alpha-cypermethrin WP & SC	PY	0.02-0.03	360	4-6	II	Cancelled
Bifenthrin WP	PY	0.025-0.05	56t	3-6	II	Active
Cyfluthrin WP	PY	0.02-0.05	250	3-6	II	Active
Deltamethrin WP, WG	PY	0.02-0.025	135	3-6	II	Active
Etofenprox WP	PY	0.1-0.3	42	3-6	U	Active
Lambda-cyhalothrin WP, CS	PY	0.02-0.03	56	3-6	II	Active

[1] (Data source: USAID, 2007; WHO, 2009).
[2] CS: capsule suspension; EC = emulsifiable concentrate; SC = suspension concentrate; WG = water dispersible granule; WP = wettable powder.
[3] OC= Organochlorines; OP= Organophosphates; C= Carbamates; PY= Pyrethroids.
[4] II: Moderately Hazardous; III: Slightly Hazardous; U: Unlikely to present acute hazard in normal use.
[5] U.S Environmental Protection Agency (EPA) registration status.

Table 4. WHO recommended insecticides for IRS against malaria vectors.[1]

2.1.1 History of DDT use in IRS

DDT (bis[4-chlorophenyl]-1,1,1-trichloroethane, or dichlorodiphenyl trichloroethane) was the first synthetic pesticide of the modern age. It promised much, but ultimately created widespread concern as an environmental hazard. It was first synthesised in 1874, and its

insecticidal properties were described by Paul Müller in the late 1930s (WHO, 1979). Commercial sales began in 1945, and DDT became widely used in agriculture to control insects, such as the pink boll worm on cotton, codling moth on deciduous fruit, Colorado potato beetle, and European corn borer. The compound was also used in sylviculture and, in a powder form, as a directly applied louse-control substance in people. In the USA, use of DDT rose until 1959 (35 771 tonnes), after which it declined gradually (11 316 tonnes in 1970) (WHO, 1979, ATSDR, 2002; Turusov et al., 2002).

DDT was the first compound used in IRS to protect people against malaria, typhus, and other insect vector-borne diseases. Its first attempt was made by the military personnel in southern Italy in 1944 and in other parts of the world in the final years of World War II (Hays, 2000). Then it was introduced as a vector control measure in civilian populations in Guyana, Venezuela, Cyprus and Sardinia (Giglioli et al., 1974; Gabaldon A, 1983). Large-scale use of DDT for disease vector control was started in 1945 (Hemingway and Ranson, 2000; Webb, 2011). The early successful campaigns of IRS with DDT against malaria vector led to the launch of the Global Malaria Eradication Campaign (GMEC) by WHO in 1955. The GMEC was based on the periodic use of IRS with DDT for 3–5 years to interrupt malaria transmission. However, weak healthcare systems, insufficient administrative, operational constraints, technical capacity, and public reaction to spraying were considered as the major factors contributing to the demise of GMEC. Also, population of anopheline resistant to DDT was primarily responsible for the dwindling political and financial support for GMEC, which ended by 1969 (Litsios, 1996).

2.1.2 Benefit of DDT use

Although GMEC did not achieve its ultimate objective, it was credited with eliminating the risk of the disease for about 700 million persons, mainly in North America, Europe, the former Soviet Union, all Caribbean islands except Hispaniola, and Taiwan (Bruce-Chwatt, 1980). In these regions, incidence of malaria was reduced to zero, or near zero (Curtis and Lines, 2000). In areas with intense and stable transmission (holoendemic to mesoendemic zones) of tropical climates, malaria vectors and prevalence rates were considerably reduced during these projects (e.g., in Cameroon, Kenya, Liberia, Nigeria, Senegal, and Tanzania) (Kouznetsov, 1977; Curtis and Mnzava, 2000; Mabaso et al., 2004). DDT is therefore credited with wholesale suppression and even complete disappearance of vector species such as *Anopheles sergenti* and *Anopheles funestus* from sizeable areas of Egypt, South Africa, Madagascar and Mauritius (Pampana, 1963; Curtis and Lines, 2000). Unfortunately, few African countries participated in the GMEC and even so, the reductions obtained were not sustained after the eradication period because limited resources were devoted to malaria control (Rogan and Chen, 2005; Sadasivaiah et al., 2007).

2.1.3 Environmental risk of DDT use

DDT is a persistent insecticide, does not occur naturally in the environment and is usually found as a white, crystalline, tasteless, almost odorless, and enters terrestrial and aquatic environments through deposition and accidental spillage. Once DDT enters the terrestrial environment, it has a strong affinity for soil and generally remains in the surface layers. As a result of this strong affinity for soil, DDT is quite a persistent pollutant. DDT has a half-life of 15 years, which means if you use 100 kg of DDT, it will break down to 0.39 kg after 120 years (Mader, 1996). This also means that after 100 years, there will still be over a pound of DDT in the environment. DDT has some potential to bio-accumulate in marine life because

it is absorbed by small organisms, such as plankton and fish. It can accumulate to high levels in fish and marine mammals (such as seals and whales), reaching levels thousands of times higher than in water. In these animals, the highest levels of DDT are found in their adipose tissue (ATSDR, 2002). With the publication of "*Silent Spring*" by Rachel Carson in 1962, the safety of DDT for human health and the environment was challenged. This was largely based on the ecological considerations, including persistence in the environment and sufficient bioaccumulation and toxic effects to interfere with reproduction in pelagic birds (i.e., thinning of eggshell). Between 1940 and 1973, estimates indicated that more than 2 million tons of DDT were used in the United States, about 80% of them in agriculture, and some level of resistance was reported in populations of 98 species of economically important insects (Metcalf, 1973). Today, no living organism may be considered free of DDT. It is stored in all tissues, but the highest concentration occurs in fats. The half-life of dichlorodiphenyldichloroethylene (DDE), a primary metabolite of DDT, is about 11 years to disappear from an individual if exposure would totally cease, but that DDE would possibly persist throughout the life span (Smith, 1991; Wolff et al., 2000).

2.1.4 Human health risk from DDT use

Toxic effects of DDT and its analogues have been extensively studied in laboratory animals. People who regularly consumed fish from the American Great Lakes were reported to have higher serum DDE concentrations (median 10 μg/L) than those who did not eat fish (5 μg/L), but they did not show impaired motor function (Schantz et al., 1999), impaired executive and visiospatial function, or reduced memory and learning capacity (Schantz et al., 2001). However, acute exposure to a high dose of DDT can cause death (Smith, 2001). Exposure to DDT or DDE increases liver weight, induces liver cytochrome P450 (CYP) 2B and 3A and aromatase (Li et al., 1995; Sierra-Santoyo et al., 2000; You et al., 2001), and causes hepatic-cell hypertrophy and necrosis (Smith, 2001). In animal, experimental studies confirmed that DDT causes hyperactivity, tremor, and seizures (Rogan and Chen, 2005). The compound is carcinogenic in non-human primates in mice and rats, mainly causing liver tumours (Takayama, 1999; Smith, 2001).

In human, DDT use has been considered generally safe. Doses as high as 285 mg/kg taken accidentally did not cause death, but such large doses led to prompt vomiting. DDT poisoning usually results in paresthesia, dizziness, headache, tremor, confusion, and fatigue (Rogan and Chen, 2005). The compound has been reported to affect neurobehavioral functions and to be associated with premature births (Van Wendel de Joode et al., 2001; Longnecker et al., 2001). Various reproductive and hormonal endpoints have been examined in both men and women, and although associations have been recorded, causal links have not been confirmed. Data from the US Collaborative Perinatal Project showed correlation between preterm delivery and raised concentration of DDE in serum (Torres-Arreola et al., 2003). It has been suggested that maternal exposure to DDT at levels known to occur from IRS could increase preterm birth and shorten duration of lactation (Rogan and Chen, 2005). With few studies mainly conducted in North America, it is difficult to predict causal relationship of DDT exposure to altered preterm delivery or duration of lactation and certainly such findings cannot be extrapolated to other settings like Africa. But if DDT does increase preterm birth and shorten lactation in Africa, it will increase infant mortality. This assumption has been seen by Rogan and Chen, (2005) abrogating the benefit of reducing infant mortality from malaria. However, better understanding on the consequence of the

increase in infant mortality from DDT exposure versus the lives saved from malaria vector control should be a matter for future research (Rogan and Chen, 2005).
Overall human health effects of DDT and DDE most commonly suggested by studies done in North America and Europe are: fertility loss, early pregnancy loss, leukemia, pancreatic cancer, neurodevelopmental deficits, diabetes, and breast cancer (Beard, 2006; Chen and Rogan, 2003; Cox et al., 2007; Eriksson and Talts, 2000; Garabrant et al., 1992; Ribas-Fito et al., 2006; Snedeker, 2001; Venners et al., 2005). In many cases the results have not been consistent between these studies, but nevertheless these accumulating reports bear much concern, particularly in relation to chronic effects. Breast cancer has been most rigorously studied; even though the majority of results showed no causative association with DDT exposure (Brody et al., 2007). This concluded that although extensively studied, there is no convincing evidence that DDT or its metabolite DDE increase risk of cancer to human (Rogan and Chen, 2005).

2.1.5 Ban of DDT use

The concerns about human health and environment led to ban of DDT in Sweden in 1970, the USA in 1972, and the UK in 1986 (Ratcliffe, 1967; Turusov et al., 2002). The global ban on DDT was proposed in 2001 when production and use of DDT are strictly restricted by an international agreement known as the Stockholm Convention on Persistent Organic Pollutants (Stockholm Convention, 2001). The Convention's objective is to protect both human health and the environment from persistent organic pollutants. DDT is one of 12 chemicals identified as a persistent organic pollutant that the Convention restricts. It has been listed in Annex B (Restriction) of the Convention and allowed to be used for disease vector control in accordance with Part II of the annex. Parties must register with the Secretariat to use DDT for disease vector control and comply with specific information collection requirements on the production and use of DDT. In May 2007, 147 countries were parties to the Convention.

2.1.6 Re-introduction of DDT use

When DDT was officially banned in the US in 1972, the WHO reported and concluded that the benefits derived from use of this pesticide were far greater than its possible risks (WHO, 1973). After 35 additional years, these benefits of DDT can be confirmed. In 2000s, several countries in sub-Saharan Africa claimed that DDT was still needed as a cheap and effective means for vector control (Turusov et al., 2002; Rogan and Chen, 2005). The Convention has given an exemption for the production and public health use of DDT for indoor application to insect vector-borne diseases, mainly because of the absence of equally effective and efficient alternatives. According to the WHO Position Statement (WHO, 2011), DDT has several characteristics that are of particular relevance in malaria vector control. Among the 12 insecticides currently recommended for IRS, DDT is the one with the longest residual efficacy when sprayed on walls and ceilings (6–12 months depending on dosage and nature of substrate). In similar conditions, other insecticides have a much shorter residual efficacy (pyrethroids: 3–6 months; organophosphates and carbamates: 2–6 months). Depending on the duration of the transmission season, the use of DDT alternatives might require more than two spray cycles per year, which would be very difficult (if not impossible) to achieve and sustain in most settings. DDT has a spatial repellency and an irritant effect on malaria vectors that strongly limit human-vector contact. Vector mosquitoes that are not directly

killed by DDT but are repelled and obliged to feed and rest outdoors, which contributes to effective disease-transmission control.

There is a general consensus that limited and strictly controlled use of DDT should be allowed for public health purposes (Liroff, 2002). This re-entering of DDT is now supported by key public health organizations and international development agencies, including the WHO, the United States Agency for International Development, and the World Bank (Hemingway and Ranson, 2000). Although the Stockholm Convention of 2001 targeted DDT as one of twelve persistent organic pollutants for phase-out and eventual elimination, it allowed a provision for its continued indoor use for disease vector control. This provision was approved without any objection by approximately 150 national delegations (Stockholm Convention on Persistent Organic Pollutants, 2001). However, still the possible adverse human health and environmental effects of exposure through IRS must be carefully weighed against the benefits of DDT as being low-cost antimalarial tool (Sadasivaiah et al., 2007). WHO has therefore approved the use of DDT under specific condition when "locally safe, effective, and affordable alternatives are not available". WHO points out that DDT spraying is "most effective in reducing the overall malaria burden in unstable transmission areas, regions with marked seasonal transmission peaks and disease outbreaks, and highlands areas" (WHO, 2004).

In general, the past decade has seen a steady increase in commitment to malaria control by the international community (Snow et al., 2008). This has caused a boost in financial and human resources available for implementation of vector control interventions, due to the support of the Global Fund, the World Bank, the U.S. President's Malaria Initiative, and many non-governmental organizations. China, the Solomon Islands, and Vietnam have largely replaced their IRS programs with ITNs during the past decades (Najera and Zaim, 2001). Conversely, the use of IRS is on the increase in Africa, where it has been more difficult to come to grips with malaria because of aspects of vector biology and disease epidemiology. IRS with DDT has become part of the national Roll Back Malaria strategic plan in several countries in Africa (Mabaso et al., 2004; Sharp et al., 2007; Hougard et al., 2002). In India, IRS with DDT has been the mainstay of vector control for more than 5 decades. In general, reports to the WHO showed that the use of DDT for malaria vector control increased substantially among the African nations during 2000–2005 (Table 5), but decreased almost to zero in the Americas due to the signing of the North American Agreement on Environmental Cooperation, a side accord to the North American Free Trade Agreement (Sadasivaiah et al., 2007).

2.2 Pyrethroids compounds used for insecticide-treated nets (ITNs)

2.2.1 History of ITNs

A mosquito net offers protection against mosquitoes, flies, and other insects, and thus against diseases such as malaria, dengue fever, yellow fever, and various forms of encephalitis, including the West Nile virus, if used properly and especially if treated with an insecticide, which can double effectiveness. The fine mesh construction stops many insects from biting and disturbing the person sleeping under net. The mesh is fine enough to exclude these insects, but it does not completely impede the flow of air. A mesh size of 1.2 mm stops mosquitoes, and smaller, such as 0.6 mm, stops other biting insects such as biting midges (no-see-ums). Mosquito netting has a long history. Though use of the term dates from the mid-18th century, use of mosquito nets has been dated to prehistoric times. It is said that Cleopatra, Queen of Egypt, also slept under a mosquito net. Mosquito nets were

Country	2003	2005	2007	Comment
Produce of DDT for vector control				
China	450	490	NA	For export
India	4,100	4,250	4,495	For malaria and leishmaniasis
DPRK	NA	NA	5	> 155 metric tons for use in agriculture
Global production	< 4,550	< 4,740	> 4,500	
Use of DDT for vector control				
Cameroon	0	0	0	Plan to pilot in 2009
China	0	0	0	Discontinued use in 2003
Eritrea	13	15	15	Epidemic-prone areas
Ethiopia	272	398	371	Epidemic-prone areas
Gambia	0	0	NA	Reintroduction in 2008
India	4,444	4,253	3,413	For malaria and leischmaniasis
DPRK	NA	NA	5	> 155 metric tons used in agriculture
Madagascar	45	0	0	Plan to resume use in 2009
Malawi	0	0	0	Plan to pilot in 2009
Mauritius	1	1	<1	To prevent malaria introduction
Morocco	1	1	0	For occasional outbreaks
Mozambique	0	308	NA	Reintroduction in 2005
Myanmar	1	1	NA	Phasing out
Namibia	40	10	40	Long-term use
Papua New Guinea	NA	NA	0	No recent use reported
South Africa	54	62	66	Reintroduction in 2000
Sudan	75	NA	0	No recent use reported
Swaziland	NA	8	8	Long-term use
Uganda	0	0	NA	High Court prohibited use, 2008
Zambia	7	26	22	Reintroduction in 2000
Zimbabwe	0	108	12	Reintroduction in 2004
Global use	> 4,953	> 5,210	> 3,950	

[1]Adapted from van den Berg, 2011.

Table 5. Annual global production and use of DDT (in 103 kg active ingredient) in 2003, 2005, and 2007.[1]

used during the malaria-plagued construction of the Suez Canal (see History of Malaria Control at: http://hub.webring.org/hub/malaria). The mosquito net, while used throughout Asia for centuries, was brought into American mainstream by Col. William Gorgas during the construction of the Panama Canal when thousands of workers, both local

and foreigner, died from the outset of malaria. Mosquito nets treated with insecticides — known as insecticide treated nets (ITNs) or bednets — were developed in the 1980s for malaria prevention (Hung et al., 2002). Newer, longer lasting insecticide nets (LLIN) are starting to replace ITN's in many countries. ITNs are estimated to be twice as effective as untreated nets and offer greater than 70% protection compared with no net (Bachou et al., 2006). These nets are treated using a synthetic pyrethroid insecticide such as deltamethrin or permethrin which improve the protection over a non-treated net by killing and repelling mosquitoes. At least 6 insecticide products are recommended by WHOPES for impregnation of mosquito nets for malaria vector control (Table 6).

Insecticides (Formulations[1])	Dosage[2]	Relevant NOAEL mg (a.i./kg bw/day)	ADI mg (safety factor of 100)	Oral toxicity LD50 (mg/kg/bw)	Dermal toxicity LD50 (mg/kg/bw)
Alpha-cypermethrin (SC 10%)	20-40	1.5	0–0.02	4,932	2,000
Cyfluthrin (EW 5%	50	2	0–0.02	2,100	>5,000
Deltamethrin (SC 1%[3])	15-25	1	0–0.01	>10,000	>10,000
Etofenprox (EW 10%)	200	3.1	0–0.03	>5,0001	>5,000
Lambda-cyhalothrin (CS 2.5%)	10-15	2.5	0-0.02	56	632
Permethrin (EC 10%)	200-500	5	0–0.05	5,000–6,000	4,000–10,000

[1]EC = emulsifiable concentrate; EW = emulsion, oil in water; CS = capsule suspension; SC= suspension concentrate; WT = water dispersible tablet.
[2]Milligrams of active ingredient per square metre of netting
[3] Formulation of WT 25%; and WT 25% + binder (K-O TAB 1-2-3®) are also recommended for this insecticide.

Table 6. WHO recommended insecticide products treatment of mosquito nets for malaria vector control.

2.2.2 Benefit of ITNs use

The use of ITNs has been shown to be an extremely cost - effective method of malaria prevention and are part of WHO's Millennium Development Goals (MDGs). These nets can often be obtained for around $2.50–$3.50 from the United Nations organizations such as WHO and UNICEF, including commercial sources, with additional cost on logistics. Generally LLIN's are purchased by donor groups like the Bill and Melinda Gates Foundation and distributed through in country distribution networks.

Studies on the cost-effectiveness of free distribution concluded on spill over benefits of increased ITN usage (Hawley et al., 2003a). ITNs not only protect the individuals or households that use them, but they also protect people in the surrounding community in several ways (Maxwell et al., 2002). First, ITNs kill adult mosquitoes, the exposure to insecticide directly increases the mortality rate and can therefore decrease the frequency in which a person is bitten by an infected mosquito (Killeen and Smith, 2007). Second, certain malaria parasites require several days to develop within the salivary glands of the vector mosquito. *Plasmodium falciparum*, the parasite responsible for the majority of deaths in sub-Saharan Africa, takes 8 days to mature and therefore malaria transmission to humans does not take place until approximately the 10th day, although would have required blood meals at intervals of 2 to 5 days (Smith and McKenzie, 2004). By killing mosquitoes prior to

maturation of the malaria parasite, ITNs can reduce the number of encounters of infected mosquitoes with humans (Killeen and Smith, 2007). When a large number of nets are distributed in one residential area, their insecticidal additives effect helps to reduce the density of mosquitoes in the environment. With fewer mosquitoes in the environment, the chances of malaria infections are significantly reduced. A review of 22 randomized controlled trials of ITNs (Lengeler, 2004) found that ITNs can reduce deaths in children by one fifth and episodes of *P falciparum* malaria by half. More specifically, in areas of stable malaria "ITNs reduced the incidence of uncomplicated malarial episodes by 50% compared to no nets, and 39% compared to untreated nets" and in areas of unstable malaria "by 62% compared to no nets and 43% compared to untreated nets". As such the review calculated that for every 1000 children protected by ITNs, 5.5 lives would be saved each year.

Despite, the wide acceptance and significant efforts made for scaling up ITNs in Africa (WHO, 2002) questions concerning the long-term acceptability and durability of this strategy are still remaining. First, reductions in all-cause child mortality rates due to short-term effect related to use of nets may not be sustainable, because initial reductions in mortality occur as a result of the combination of reduced malaria transmission and pre-existing partial immunity developed under the formerly higher levels of transmission. After transmission declines and immunity wanes, mortality rates may increase (Molineaux, 1997). Second, pyrethroid resistance in Anopheles mosquitoes might compromise the long-term effectiveness of ITNs in killing mosquitoes (Zaim and Guillet, 2002). Third, it is not clear whether the community will maintain proper use of nets and sustain (adherence) over long periods, particularly when nets are distributed free of charge (Curtis et al., 2003). Fourth, acquired immunity against clinical malaria, a function of the frequency of infections, is delayed as it is developing gradually with time. Therefore, the period during which a child is at risk from clinical malaria might increase where ITNs are used (Snow and Marsh, 1995; Trape and Rogier, 1996). The practical impact of this hypothesis is that: if a child was protected by ITNs but later these were no longer provided or were not used, there might be a rebound effect of clinical disease when the child is exposed to infectious mosquitoes.

Some of carefully controlled efficacy trials that have been running up to 6 years period have shown the benefit of using ITNs in Africa. Results of research project in western Kenya, using randomized controlling trials, showed that ITNs use led to: First, 90% reductions in malaria vector population (Gimnig et al., 2003), 74% reduction in force of infection in infants (ter Kuile et al., 2003a), and 23% reduction in all-cause mortality in infants (excluding neonates) (Phillips-Howard et al., 2003a). Second, no evidence for compromised immunologic antibody response has been confirmed in children less than five years of age (Kariuki et al., 2003). Third, clear beneficial effects on malaria specific morbidity (clinical malaria, malarial anemia) and growth in infants and 1–3 year-old children have been confirmed. Fourth, reduction in exposure to malaria in infancy does not, with continued use of nets for 22 months, result in increased malaria morbidity in one-year-old children (ter Kuile et al., 2003a & b). Fifth, clear reduction in visits of sick children to health facilities associated with ITNs use with concomitant reduction in quantities of antimalarial drugs prescribed (Phillips-Howard et al., 2003b). Sixth, clear benefits associated with pregnancy, including reduced maternal and placental malaria, maternal anemia, and low birth weight (for the first four pregnancies) (ter Kuile et al., 2003b). Seventh, beneficial effects of ITNs spill over into areas adjacent to villages with ITNs; magnitude of this community mass effect is similar to that observed within ITNs villages and dependent upon coverage i.e. the proportion of houses in a given area with ITNs (Hawley et al. 2003; Gimnig et al., 2003). Eighth, evidence for the existence of a community-wide effect due

to marked reduction in vector populations (Howard et al., 2000; Hii et al., 2001; Maxwell et al., 2002), implying that ITNs have substantial effects at the population level. Finally, all these public health benefits of ITNs were sustained for up to 6 years and there is no evidence that bed-net use from birth increases all-cause mortality in older children (Lindblade et al. 2004). All these findings have been demonstrated in areas under setting of intense perennial malaria transmission. More recently, Fegana et al. (2007) associated ITNs use (67% coverage), under different settings of malaria transmission in Kenya, with 44% reduction in mortality in children less than five years.

3. Hazard of pyrethroids insecticides use for ITNs and IRS

Massive use of ITNs began in 1980s following the developmental of photostable synthetic pyrethroids which are faster acting, effective in small quantities, relatively stable adhering to fabric, and relatively safe to human (WHO,1999). Scale up of the ITNs usage has emerged as a key intervention for malaria control in 2000s. The initial aim of Roll Back Malaria (RBM) was to cover 60% of population in malaria endemic countries, which was refined to achieve coverage of 2 bed nets per household. In this case millions of people were expected to be exposed at different dosages of pyrethroids in malaria endemic countries. Washing large quantities of ITNs leading to spill over of insecticide to water bodies could be hazardous to both human and aquatic environment. Likewise regular re-treatment and use of nets as well as use of LLIN's increases the risk of acute toxicity among net dippers and regular users. Also new technology with potential for malaria prevention, such as insecticide impregnated durable wall lining (DL), insecticide treated blankets and tents (e.g. Demuria nets) pre-treated at the factory with high concentration of insecticide, increase the risk of acute toxicity to people doing installation and household occupants coming into contact. In one of WHO's statements regarding the safety of pyrethroid treated mosquito nets (WHO, 1999), it was asserted that if prescribed precautions are followed, field use of these products at concentrations recommended for treatment of mosquito nets poses little or no hazard to people treating the nets or to users of the treated nets. Although other risk assessment of the use of deltamethrin on ITNs largely supports this view of the WHO, a relatively high chronic risk (beyond the US EPA standard of 0.01 mg. active ingredient/kg/body weight) was shown to exist for newborns sleeping under ITNs (Barlow et al., 2001).

All pesticides are toxic by nature and present risks of adverse effects that depend on toxicity of the chemical and the degree of exposure. Toxicity refers to the inherent poisonous potency of a compound under experimental conditions, and chronic toxicity refers to the potential for adverse effects from long-term exposure (Hirsch et al., 2002). While there is agreement that ITNs can be effective in reducing malaria morbidity and mortality under field trials, the adverse effects associated with their use at different level of age groups and sex has not yet to be fully evaluated. Some scientists raised concerns about the long-term effects of ITNs exposures, especially on children and pregnant women (Anyanwu et al., 2004). In their comprehensive literature review, Anyanwu et al. (2006) show that not much work has been done on the effects of long-term exposure to ITNs. But the authors surprisingly concluded that the results of their search on the subject to date seem to support only the efficacy of the temporal use of plain bed nets, but not the use of ITNs, and do not tell much about the long-term effects of ITNs exposure (Anyanwu et al., 2006). Indeed, all pesticides are toxic and have both acute and chronic effects (Ratnasooriya et al., 2003). While there is no doubt about the effectiveness of ITNs and the main challenge now is to scale up

their use (WHO, 2002). Review reports on the benefits of ITNs did not yield any information relating to the potential adverse effects of long-term exposure to insecticide treated products (Anyanwu et al., 2006). However, Kolaczinski and Curtis (2004) concluded that chronic effects can presently not be excluded with certainty, as relevant toxicological data do not exist in the open scientific literature. Properly designed neuro-behavioural studies on groups with long-term exposure to low doses of synthetic pyrethroids should be conducted in order to assess effect of exposure of ITN's. Meanwhile pyrethroids should continue to be used for public health interventions to contribute reducing malaria morbidity and mortality reduction, such as ITNs for malaria control.

On the other hand, IRS insecticides applied indoors of dwellings is subject to a number of considerations and constraints. Similar constraints should apply to new technology under evaluation, such as the durable wall lining (DL) impregnated with high concentration of insecticide, with characteristic of both IRS and LLIN. One of these considerations relates to the required residual effectiveness of the insecticide applied to last the malaria transmission season (Table 4). It is therefore logical that active ingredients (AIs) used in IRS and DL should be biologically available to control the mosquito vectors, but also at the same time potentially available for human uptake via various routes. These routes conceivably include dermal uptake, inhalation (dust and gas phase), and ingestion. As pointed out elsewhere, there probably exists a dynamic redistribution of applied insecticide through a continuous process of indoor sublimation, deposition, and revolatilization, as well as dust movement, necessitating a total home stead environment approach when considering exposure (Sereda et al. 2009). Bouwman and Kylin (2009) showed that infants under malaria control conditions are exposed to combinations of chemicals that would have deleterious effects if the intakes were high enough. They actually showed that the intakes through breast milk do exceed acceptable levels of intake, but they do not attributed the whole level of exposure to insecticides used in malaria control i.e. agricultural and home garden use could also contribute to the levels in the tissue and in breast milk. Generally, the possible resultant toxicity from this exposure could be attributable to either a single compound or combinations of several that could act additively, antagonistically, independently, or possibly synergistically. Critical windows of exposure also need to be considered. The health effects might be transient, reversible, latent, and/or permanent, and might also be subtle and not readily attributable to insecticide use for vector control. Given that IRS and ITNs also effectively reduce morbidity and mortality of malaria, this resulting in a paradox that is a characteristic of many situations where risks and positive outcomes need to be measured and balanced. Because millions of people in malaria control areas experience conditions of multiple sources and routes of exposure to any number of insecticides, even though lives are saved through malaria prevention, identification of potential health risks to infant associated with insecticide residues in breast milk must be incorporated in WHOPES evaluations and in the development of appropriate risk assessment tools (Bouwman and Kylin 2009).

4. Insecticide resistance in insect vectors

Much of the available insecticides for vector control, which have been spectacularly successful in the past, are more than 35 years old (Table 7). For example, early efforts to control malaria during the 1950s and 1960s with spraying indoors with DDT and other insecticides achieved almost total eradication of the vector and the pathogen in many parts of the world (Gramiccia and Beales, 1988; Mabaso et al., 2004; Roberts et al., 2000). These

efforts simultaneously reduced levels of transmission of dengue, leishmaniasis and filariasis. Some countries, such as Taiwan, are now celebrating 40 transmission-free years of malaria. This is a massive achievement, as malaria was previously a major killer in the country (Hemingway et al., 2006). More recently, ITNs reduced morbidity and also mortality from all causes (Phillips-Howard et al., 2003a; Lengeler, 2004). This is a result of protection at the levels of the individual and the community (Lindblade et al., 2004). Control of dengue vectors relies on the removal of larval breeding containers, such as old tyres or flower vases or on insecticide spraying in homes. This approach has been used successfully in some locations, but is not sustainable (Rigau-Perez et al., 2002; Gubler, 1989). Due to insecticide resistance, legitimate environmental and human health concerns, the use of many older generation insecticides, such as DDT is decreasing. The result is that the number of public health insecticides available is dwindling and vector-borne disease transmission is increasing (Hemingway et al., 2006).

Resistance is defined as a heritable change in the sensitivity of a population to an insecticide, which is reflected in the repeated field failure of that product to achieve the expected level of control when used according to the recommendations for that pest species, and where problems of product storage, application and unusual climatic or environmental conditions can be eliminated (McCaffery and Nauen, 2006). Frequent applications of the same insecticide will select for those individuals in a population, with inherent genetic advantage, that are able to survive the recommended dose of the compounds. Over time, this selection pressure will lead to a resistant population becoming established. In such cases, other compounds within the same class of chemistry are in most cases also affected – for instance, resistance to one pyrethroid type usually confers resistance against the whole group of pyrethroids, a phenomenon known as cross-resistance. Sometimes, depending on the nature of the resistance mechanism, cross-resistance can occur between different chemical classes, for example organophosphates and carbamates, and cross resistance between DDT and pyrethroids (multi-resistance). Furthermore, resistance development due to selection pressure in disease vectors is sometimes complicated by an additional (perhaps sometimes neglected) aspect: the frequent application of similar synthetic insecticides to control pests of agricultural importance. This may indirectly affect the susceptibility of insects of public health importance, because that is where the vectors are additionally exposed to pesticides used for agricultural purpose (Brogdon and McAllister, 1998; Liu et al., 2006; Hemingway and Ranson, 2000; Nauen, 2007).

4.1 Insecticide resistance mechanisms

Four classes of chemical insecticides are the mainstay of vector control programmes: namely organochlorines, organophosphates, carbamates, and pyrethroids (WHO, 2006a). To date, four types of resistance mechanisms against the chemical insecticides have been described: metabolic resistance, target site resistance, penetration resistance, and behavioural resistance. Metabolic and target site resistance have been extensively investigated at both the genetic and molecular levels (Hemingway and Ranson, 2000). Metabolic resistance involves the sequestration, metabolism, and/or detoxification of the insecticide, largely through the overproduction of specific enzymes (Hemingway and Karunaratne, 1998; Hemingway et al., 1998). So far, three main groups of enzymes have been identified in different insect vectors species (Table 7): carboxylesterases (EST: efficient against organophosphate and carbamate insecticides), glutathione- S-transferases (GST: efficient against organophosphates, organochlorine, and pyrethroid insecticides) and cytochrome P450-dependent monoxygenases (MOX: efficient against most insecticide types, frequently

Years	WHO approved insecticides			Comments
1940-45	DDT			
1946-50	Lindane			
1951-55	Malathion			Only a limited number of insecticide classes are available for insect vectors control. No new insect vector adulticide has been approved by the WHO the last 20 years.
1956-60				
1961-65	Fenitrothion	Propoxure		
1966-70	Chlorpyrifose-methyl			
1971-75	Pirimiphose-methyl	Bendiocarb	Permethrin	
1976-80	Cypermethrin			
1981-85	Alpha-cypermethrin	Cyfluthrin		
	Lambada-cyhalothrin	Deltamethrin	Bifenthrin	
1986-90	Etofenprox			
1991-95				
1996-00				
2001-05				
2006-10				
	Organochlorines		Carbamates	
	Organophosphates		Pyrethroids	

[1]Adapted from Nauen, 2007.

Table 7. History of WHO-approved insecticides for adult malaria mosquito control.[1]

in conjunction with other enzymes). The overproduction of these enzymes may be achieved via two nonexclusive mechanisms: gene amplification increasing the gene's copy number (Hemingway et al., 1998) and gene expression via modifications in the promoter region or mutations in trans-acting regulatory genes (Hemingway et al., 1998; Rooker et al., 1996). In addition, in some mosquito species, carboxylesterase resistance to the insecticide malathion has been associated with a qualitative change in the enzyme (a few amino acid substitutions can increase the rate of hydrolysis of the enzyme (Hemingway et al., 2004). In contrast, target site resistance is achieved by point mutations that render the actual targets of an insecticide less sensitive to the active ingredient (Hemingway and Ranson, 2000; Weill et al., 2003). Most insecticides developed to date are neurotoxic and aim for one of the following three targets: the acetylcholinesterase (AChE) (whose role is the hydrolysis of the neurotransmitter acetylcholine), the c-aminobutyric acid (GABA) receptors (chloride-ion neurotransmission channels in the insect's nervous system), or the sodium channels (responsible for raising the action potential in the neurons during the nerve impulses). The acetylcholinesterase is the target of organophosphorous and carbamate insecticides, the GABA receptors are the main targets of cyclodiene (organochlorine) insecticides, and the sodium channels (resistance by modification of this site known as knockdown resistance

(KDR)) are the targets of pyrethroid and organochlorine insecticides. Mutations in all these three sites can confer resistance (Table 8).

More recently, two alternative insecticide types have been introduced, largely for the control of mosquito larvae: bio-pesticides (e.g., Bacillus thuringiensis, Bacillus sphaericus) and insect growth regulators, such as the juvenile hormone mimic and methoprene (WHO, 2006a). Cases of resistance to these alternative insecticides are still limited (Rivero et al., 2010) and the underlying mechanisms are only beginning to be identified (Chalegre et al., 2009; Darboux et al., 2007).

Vector	Pathogen (Disease)	Insecticide Resistance	
		Metabolic	Target Site
Diptera (mosquitoes, flies)			
Aedes sp.	Brugia, Wuchereria (lymphatic filariasis), yellow fever virus, dengue virus, encephalitis virus	EST	KDR
		GST	GABA
Anopheles sp.	Plasmodium sp. (malaria), Wuchereria (filariasis)	EST	KDR
		GST	AChE
		MOX	GABA
Culex sp.	Wuchereria (filariasis), West Nile virus, encephalitis virus	EST	KDR
		GST	AChE
		MOX	GABA
Phlebotomus sp.	Leishmania sp. (leishmaniasis)	EST	AChE
Simulium sp.	Onchocerca sp. (river blindness)	EST	-
Haemiptera (true bugs)			
Rhodnius sp.	Trypanosoma sp. (Chagas disease)	?	?
Triatoma sp.	Trypanosoma sp. (Chagas disease)	EST MOX	-
Phiraptera (body lice)			
Pediculus sp.	Rickettsia sp. (epidemic thyphus)	?	?
Siphonaptera (fleas)			
Xenopsylla sp.	Pasturella (bubonic plague)	?	?

[1]Adapted from Rivero et al. (2010).
Metabolic resistance: EST, enhanced esterase activity; GST, enhanced glutatione-S-transferase activity; MOX, enhanced p450 monoxygenase activity. Target site resistance: AChE, modification of the acetylcholinesterase; GAB, modification of the GABA receptors; KDR, (knockdown resistance) modification of the sodium channels. ?, Insecticide resistance present but mechanism unknown or unconfirmed to the best of our knowledge.

Table 8. Insecticide resistance mechanisms reported to date in natural populations of the main insect vectors of human diseases[1].

4.2 Resistance and disease control

To compromise insecticide vector control, the level of resistance must be high enough to adversely affect disease transmission. In many cases, vector control may not be affected by the level of resistance. For example, an activity may be controlling only 75% of the vector population. If, for example, the level of resistance is lower than 10%, resistance will

not affect disease control efforts; in this situation, increasing surveillance and monitoring level and frequency of resistance would be sufficient. No change in control methods would be needed (Brogdon and McAllister, 1998). Western Kenya is a good operational example of the coexistence of resistance and disease control. Pyrethroid resistance appeared soon after bed nets were introduced in Kenya. After 2 years, the resistance level had not changed significantly, possibly because of the continual introduction of susceptible genes (Vulule et al., 1996). Other reasons may explain why the presence of insecticide resistance genes in vectors in a control area does not mean that effective control is not being achieved. For example, resistance genes may not be expressed, they may be expressed in an alternative stage of development to that being controlled by insecticide, or the gene detected may be a member of an alternative gene subfamily to one that can affect the compound being used (Brogdon and McAllister, 1998). For example, in *An. albimanus,* resistance enzymes, especially esterases and GST, may be expressed only in freshly emerged adult anophelines and may be absent in older mosquitoes, those potentially infectious for malaria (Brogdon et al., 1999). In six populations of *An. arabiensis* from Sudan, the L1014F-kdr resistance allele present in 66% dead individuals against the WHO discriminating concentrations of permethrin (Himeidan et al., 2011) suggesting that another factor in the para-type sodium channel gene might be needed for the expression of kdr resistance phenotype (Brooke, 2008).

Insecticide resistance is viewed as an extremely serious threat to crop protection and vector control, and is considered by many parties, including industry, the WHO, regulatory bodies and the public, to be an issue that needs a proactive approach. In 1984, the Insecticide Resistance Action Committee (IRAC) was formed in order to provide a coordinated private-sector response to prevent or at least delay the development of resistance (www.irac-online.org) (McCaffery and Nauen, 2006).

The Innovative Vector Control Consortium (IVCC) was formed in 2005, with an initial grant of $50.7 million from Bill & Melinda Gates Foundation over five years, as a new initiative to enable industry and academia to join forces to improve the portfolio of chemical and technological tools available to reduce vector-borne diseases. Since then, an unprecedented development pipeline of new, reformulated and repurposed insecticides has been established in partnership projects with leading global chemical companies. A suite of information systems and diagnostic tools for the more effective and efficient use of insecticides has also been developed, with these products now nearing the end of their development phase and being readied for rollout in the coming year. Accordingly, IVCC has received another $50 million in 2010 from the Bill & Melinda Gates Foundation to continue its work to develop new insecticides for the improved control of mosquitoes and other insects which transmit malaria, dengue and other neglected tropical diseases. As resistance to insecticides is increasing at an alarming rate and it must find new alternatives insecticides against malaria vectors and other vector borne diseases, the strategic aim of IVCC is to provide three new Active Ingredients for use in public health insecticides by 2020.

5. Authors' contributions

YEH identified the idea, drafted and wrote up the chapter, EJK and EAT critically reviewed the content and proof read the chapter. All authors read and approved the final chapter.

6. References

Ameneshewa B, Dash AP, Ehrenberg J, Ejov M, Frederickson C, Jambulingam P, Mnzava A, Prasittisuk C, Velayudhan R, Yadav R, Zaim M, (2009). Global Insecticide Use for Vector-Borne Disease Control. World Health Organization Pesticide Evaluation Scheme (WHOPES). Geneva: World Health Organization. Available at: http://whqlibdoc.who.int/publications/2009/9789241598781_eng.pdf

Anyanwu EC, Ehiri JE, Kanu I, Merrick J, (2006). Health effects of long-term exposure to insecticide-treated mosquito nets in the control of malaria in endemic regions, revised. ScientificWorldJournal. 6:1631-41.

Anyanwu EC, Ehiri JE, Kanu I, Morad M, Ventegodt S, Merrick J. Assessing the health effects of long-term exposure to insecticide-treated mosquito nets in the control of malaria in endemic regions. ScientificWorldJournal. 2004 Nov 19;4:978-88.

ATSDR, (2002). (Agency for Toxic Substances and Disease Registry). Toxicological profile for DDT/DDD/DDE (update): US Department of Health and Human Services. Public Health Service. Atlanta, GA: Agency for Toxic Substances and Disease Registry, 2002.

Bachou H, Tylleskär T, Kaddu-Mulindwa DH, Tumwine JK (2006). Bacteraemia among severely malnourished children infected and uninfected with the human immunodeficiency virus-1 in Kampala, Uganda". BMC Infect. Dis. 6: 160.

Barlow SM, Sullivan FM, Lines J. Risk assessment of the use of deltamethrin on bednets for the prevention of malaria. Food and Chemical Toxicology 2001; 39:407–422.

Beard J. DDT and human health. Sci Total Environ 2006; 355:78-89.

Beier JC, Keating J, Githure JI, Macdonald MB, Impoinvil DE, Novak RJ, 2008. Integrated vector management for malaria control. Malar J, 7 Suppl 1:S4.

Bomann,W., 1995. How safe are pyrethroid-treated mosquito nets? An evaluation based on the example of Solfac EW 050. Public Health 12,30–35.

Bouwman H, Kylin H. 2009. Malaria control insecticide residues in breast milk: the need to consider infant health risks. Environ Health Perspect;117(10):1477-80.

Brody JG, Moysich KB, Humblet O, Attfield KR, Beehler GP, Rudel RA. Environmental pollutants and breast cancer: epidemiologic studies. Cancer 2007; 109(Suppl.12):2667-2711.

Brogdon WG and McAllister JC, Insecticide resistance and vector control. Emerg Infect Dis 4:605–613 (1998).

Brogdon WG, McAllister JC, Corwin AM, Cordon-Rosales C. 1999, Independent selection of multiple mechanisms for pyrethroid resistance in Guatemalan Anopheles albimanus (Diptera: Culicidae). J Econ Entomol.;92(2):298-302.

Brooke BD. 2008. kdr: can a single mutation produce an entire insecticide resistance phenotype? Trans R Soc Trop Med Hyg. 2008 Jun;102(6):524-5.

Bruce-Chwatt LJ, (1980). *Essential Malariology*. London: Heinemann Medical Books Ltd.

Chalegre KD, Romão TP, Amorim LB, Anastacio DB, de Barros RA, de Oliveira CM, Regis L, de-Melo-Neto OP, Silva-Filha MH. (2009) Detection of an allele conferring resistance to Bacillus sphaericus binary toxin in Culex quinquefasciatus populations by molecular screening. Appl Environ Microbiol 75: 1044–1049.

Chen A, Rogan WJ. Nonmalarial infant deaths and DDT use for malaria control. Emerg Infect Dis 2003; 9:960-964.

Cohn BA, Wolff MS, Cirillo PM, Sholtz RI. DDT and breast cancer in young women: new data on the significance of age at exposure. Environ Health Perspect 2007; 115:1406-1414.

Cox S, Niskar AS, Narayan KM, Marcus M. Prevalence of self-reported diabetes and exposure to organochlorine pesticides among Mexican Americans: Hispanic Health and Nutrition Examination Survey, 1982-1984. Environ Health Perspect 2007; 115:1747- 1752.

Curtis CF, Lines JD, (2000). Should DDT be banned by international treaty? Parasitol Today 16: 119-21 .

Curtis CF, Maxwell CA, Lemnge M, Kilama WL, Steketee RW, Hawley WA, Bergevin Y, Campbell CC, Sachs J, Teklehaimanot A, et al. (2003). Scaling-up coverage with insecticide-treated nets against malaria in Africa: who should pay? *Lancet Infect Dis* 3: 304-307.

Curtis CF, Mnzava AE, (2000). Comparison of house spraying and insecticide-treated nets for malaria control. Bull World Health Organ 78: 1389–1400.

Darboux I, Charles JF, Pauchet Y, Warot S, Pauron D (2007) Transposonmediated resistance to Bacillus sphaericus in a field-evolved population of Culex pipiens (Diptera : Culicidae). Cell Microbiol 9: 2022–2029.

Eriksson P, Talts U. Neonatal exposure to neurotoxic pesticides increases adult susceptibility: a review of current findings. Neurotoxicology 2000; 21:37-47.

Fegana GW, Noora AM, Akhwalec WS, Cousensb S, Snow RW, (2007). Effect of expanded insecticide-treated bednet coverage on child survival in rural Kenya: a longitudinal study. *Lancet* 370: 1035–39.

Gabaldon A, (1983). Malaria eradication in Venezuela: doctrine, practice, and achievements after twenty years. *Am J Trop Med Hyg 32:* 203–211.

Garabrant DH, Held J, Langholz B, Peters JM, Mack TM. DDT and related compounds and risk of pancreatic cancer. J Natl Cancer Inst 1992; 84:764-771.

Giglioli G, Wan-I C, Howell P, Marchant D, (1974). Malaria eradication under continental equatorial conditions in Guyana. *West Indian Med J 23:* 25–34.

Gimnig JE, Kolczak MS, Hightower AW, Vulule JM, Schoute E, Kamau L, Phillips-Howard PA, ter Kuile FO, Nahlen BL, Hawley WA, (2003a). Effect of permethrin-treated bed nets on the spatial distribution of malaria vectors in western Kenya. *Am J Trop Med Hyg* 68: 115–120.

Gramiccia, G. and Beales, P. (1988) The recent history of malaria control and eradication. In Malaria: Principles and Practices of Malariology (Wernsdorfer, W.H. and MacGregor, I., eds), pp. 1335–1378, Churchill

Gubler, D.J. (1989) Aedes aegypti and Aedes aegypti-borne disease control in the 1990s: top down or bottom up. Am. J. Trop. Med. Hyg. 40, 571–578

Hawley WA, Phillips-Howard PA, ter Kuile FO, Terlouw DJ, Vulule JM, Ombok M, Nahlen BL, Gimnig JE, Kariuki SK, Kolczak MS, Hightower AW, (2003a). Community-wide effects of permethrin-treated bed nets on child mortality and malaria morbidity in western Kenya. Am J Trop Med Hyg 68 (Suppl 4): 121–127.

Hemingway J and Bates I, (2003). Malaria: past problems and future prospects. *EMBO Rep* 4:29–31.

Hemingway J, Hawkes N, Prapanthadara LA, Jayawardenal KGI, Ranson H (1998) The role of gene splicing, gene amplification and regulation in mosquito insecticide resistance. Philos Trans R Soc Lond B Biol Sci 353: 1695–1699.

Hemingway J, Hawkes NJ, McCarroll L, Ranson H (2004) The molecular basis of insecticide resistance in mosquitoes. Insect Biochem Mol Biol 34: 653–665.

Hemingway J, Karunaratne S (1998) Mosquito carboxylesterases: a review of the molecular biology and biochemistry of a major insecticide resistance mechanism. Med Vet Entomol 12: 1–12.

Hemingway J, Ranson H (2000) Insecticide resistance in insect vectors of human disease. Annu Rev Entomol 45: 371–391.

Hii JL, Smith T, Vounatsou P, Alexander N, Mai A, Ibam E, Alpers MP, (2001). Area effects of bednet use in a malaria-endemic area in Papua New Guinea.*Trans R Soc Trop Med Hyg* 95:7-13.

Himeidan YE, Abdel Hamid MM, Jones CM, Ranson H. 2011. Extensive permethrin and DDT resistance in *Anopheles arabiensis* from eastern and central Sudan. Parasit Vectors. 4; 154

Hirsch B, Gallegos C, Knausenberger W, Arata A. Programmatic environmental assessment for insecticide-treated materials in USAID activities in sub-Saharan Africa. Agency for International Development (USAID), Office of Sustainable Development. 2002. http://www.afr-sd.org/documents/iee/docs/32AFR2_ITM_PEA.doc

Hougard JM, Fontenille D, Chandre F, Darriet F, Carnevale P, Guillet P, (2002). Combating malaria vectors in Africa: current directions of research. *Trends Parasitol* 18: 283–86.

Howard SC, Omumbo J, Nevill C, Some ES, Donnelly CA, Snow RW, (2000). Evidence for a mass community effect of insecticide- treated bednets on the incidence of malaria on the Kenyan coast. *Trans R Soc Trop Med Hyg* 94: 357–360.

Hung le Q, Vries PJ, Giao PT, Nam NV, Binh TQ, Chong MT, Quoc NT, Thanh TN, Hung LN, Kager PA (2002) Control of malaria: a successful experience from Viet Nam. Bull World Health Organ 80: 660–666.

Kariuki SK, Lal AA, Terlouw DJ, ter Kuile FO, Ong'echa JMO, Phillips-Howard PA, Orago ASS, Kolczak MS, Hawley WA, Nahlen BL, Shi YP, (2003). Effects of permethrin-treated bed nets on immunity to malaria in western Kenya. II. Antibody responses in young children in an area of intense malaria transmission. *Am J Trop Med Hyg* 68: 10–15.

Killeen GF, Smith TA (2007). Exploring the contributions of bed nets, cattle, insecticides and excitorepellency to malaria control: a deterministic model of mosquito host-seeking behaviour and mortality. *Trans R Soc Trop Med Hyg*. 2007 Sep;101(9):867-80.

Kolaczinski JH, Curtis CF, 2004. Chronic illness as a result of low-level exposure to synthetic pyrethroid insecticides: a review of the debate. Food Chem Toxicol, 42(5):697-706

Kouznetsov RL, (1977). Malaria control by application of indoor spraying of residual insecticides in tropical Africa and its impact on community health. *Trop Doct 7:* 81–91.

Lengeler C, (2004). Insecticide-treated bed nets and curtains for preventing malaria. *The Cochrane Database of Systematic Reviews* 2:CD000363.pub2.

Li HC, Dehal SS, Kupfer D. Induction of the hepatic CYP2B and CYP3A enzymes by the proestrogenic pesticide methoxychlor and by DDT in the rat. Effects on methoxychlor metabolism. *J Biochem Toxicol* 1995; 10: 51–61.

Lindblade KA, Eisele TP, Gimnig JE, Alaii JA, Odhiambo F, ter Kuile FO, Hawley WA, Wannemuehler KA, Phillips-Howard PA, Rosen DH, Nahlen BL, Terlouw DJ, Adazu K, Vulule JM, Slutsker L, (2004). Sustainability of reductions in malaria transmission and infant mortality in western Kenya with use of insecticide-treated bednets: 4 to 6 years of follow-up. *JAMA* 291:2571-2580.

Litsios S, (1996). *The Tomorrow of Malaria.* Wellington, New Zealand: Pacific Press.

Liu N, Xu Q, Zhu F and Zhang L, Pyrethroid resistance in mosquitoes. Insect Sci 13:159–166 (2006).

Longnecker MP, Klebanoff MA, Zhou H, Brock JW, (2001). Association between maternal serum concentration of the DDT metabolite DDE and preterm and small-for-gestational- age babies at birth. *Lancet* 358:110–114.

Mabaso LHM, Sharp B, Lengeler C, (2004). Historical review of malarial control in southern Africa with emphasis on the use of indoor residual house-spraying. *Trop Med Int Health* 9: 846– 856.

Mader, SS. 1996. *Biology* - 5th Ed. WCB and Cox, G.W. 1997. *Conservation Biology* - 2nd ed. WCB

Maxwell CA, Msuya E, Sudi M, Njunwa KJ, Carneiro IA, Curtis CF, (2002). Effect of community-wide use of insecticide-treated nets for 3-4 years on malarial morbidity in Tanzania. *Trop Med Int Health* 7: 1003−1008.

McCaffery A and Nauen R, The Insecticide Resistance Action Committee (IRAC): public responsibility and enlightened industrial self interest. *Outlook Pest Manag* 17:11–14 (2006).

Metcalf RL, (1973). A century of DDT. *J Agric Food Chem 21:* 511–519.

Molineaux L, (1997). Malaria and mortality: some epidemiological considerations. *Ann Trop Med Parasitol* 91:811-825.

Najera JA, Zaim M. (2001). Malaria vector control: insecticides for indoor residual spraying. WHO/CDS/ WHOPES/2001.3. Geneva: World Health Organization; 2001.

Nauen R. 2007. Insecticide resistance in disease vectors of public health importance. Pest Manag Sci. 2007 Jul;63(7):628-33.

Pampana E, 1963. A textbook of malaria eradication. London: Oxford University Press.

Phillips-Howard PA, Nahlen BL, Kolczak MS, Hightower AW, ter Kuile FO, Alaii JA, Gimnig JE, Arudo J, Vulule JM, et al., (2003a). Efficacy of permethrin-treated bed nets in the prevention of mortality in young children in an area of high perennial malaria transmission in western Kenya. *Am J Trop Med Hyg 68 (Suppl 4):* 23–29.

Phillips-Howard PA, Nahlen BL, Wannemuehler KA, Kolczak MS, ter Kuile FO, Gimnig JE, Alaii JA, Odacha A, Vulule JM, Hawley WA, (2003b). Impact of permethrin-treated bed nets on the incidence of sick child visits to peripheral health facilities. *Am J Trop Med Hyg 68 (Suppl 4):* 38–43.

Ranson H, N'guessan R, Lines J, Moiroux N, Nkuni Z, Corbel V, 2011. Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? Trends in Parasitology February 2011, Vol. 27, No. 2

Ratcliffe DA, (1967). Decrease in eggshell weight in certain birds of prey. *Nature* 215: 208–10.

Ribas-Fito N, Torrent M, Carrizo D, Munoz-Ortiz L, Julvez J, Grimalt JO, Sunyer J. In utero exposure to background concentrations of DDT and cognitive functioning among preschoolers. Am J Epidemiol 2006; 164:955-962.

Rigau-Pérez JG, Ayala-López A, García-Rivera EJ, Hudson SM, Vorndam V, Reiter P, Cano MP, Clark GG. (2002) The reappearance of dengue-3 and a subsequent dengue-4 and dengue–1 epidemic in Puerto Rico in 1998. Am. J. Trop. Med. Hyg. 67, 355–362.

Rivero A, Vézilier J, Weill M, Read AF, Gandon S, 2010. Insecticide control of vector-borne diseases: when is insecticide resistance a problem? PLoS Pathog 2010, 5:6(8).

Roberts DR, Manguin S, Mouchet J. (2000) DDT house spraying and re-emerging malaria. Lancet 356, 330–332

Roberts, L. and Enserink, M. (2007) Malaria. Did they really say. . .eradication? Science 318, 1544–1545

Rogan WJ, Chen A. Health risks and benefits of bis (4-chlorophenyl)-1,1,1- trichloroethane (DDT). *Lancet* 2005; 366: 763–73

Rooker S, Guillemaud T, Berge J, Pasteur N, Raymond M (1996) Coamplification of esterase A and B genes as a single unit in Culex pipiens mosquitoes. Heredity 77: 555–561.

Sadasivaiah S, Tozan Y, Breman JG, 2007. Dichlorodiphenyltrichloroethane (DDT) for indoor residual spraying in Africa: how can it be used for malaria control? Am J Trop Med Hyg, 77(6 Suppl):249-63.

Schantz SL, Gardiner JC, Gasior DM, Sweeney AM, Humphrey HE, McCaffrey RJ. Motor function in aging Great Lakes fisheaters. Environ Res 1999; 80: S46–S56.

Schantz SL, Gasior DM, Polverejan E, et al. Impairments of memory and learning in older adults exposed to polychlorinated biphenyls via consumption of Great Lakes fish. Environ Health Perspect 2001; 109: 605–11.

Sereda B, Bouwman H, Kylin H. 2009. Comparing water, bovine milk, and indoor residual spraying as possible sources of DDT and pyrethroid residues in breast milk. J Toxicol Environ Health A 72(13):842–851.

Sharp BL, Ridl FC, Govender D, Kuklinski J, Kleinschmidt I, (2007). Malaria vector control by indoor residual insecticide spraying on the tropical island of Bioko, Equatorial Guinea. *Malar J* 6:52.

Shousha AT (1948). The eradication of Anopheles gambiae from upper Egypt 1942-1945. Bull Wld Hlth Org 1:309-352

Sierra-Santoyo A, Hernandez M, Albores A, Cebrian ME. Sexdependent regulation of hepatic cytochrome P-450 by DDT. *Toxicol Sci* 2000; 54: 81–87.

Smith AG, (1991). Chlorinated hydrocarbon insecticides. In: Handbook of Pesticides Toxicology (Hayes WJ, Laws ER, eds). San Diego/New York: Academic Press Inc.,pp. 731–915.

Smith AG, (2001). DDT and its analogs. In: Krieger R, ed. Handbook of pesticide toxicology. 2nd edn. San Diego, CA, USA: Academic Press: 1305–55.

Smith DL, McKenzie FE (2004) Statics and dynamics of malaria infection in Anopheles mosquitoes. Malaria Journal 3: 13.

Snedeker SM. Pesticides and breast cancer risk: a review of DDT, DDE, and dieldrin. Environ Health Perspect 2001; 109:35-47.

Snow RW, Guerra CA, Mutheu JJ, Hay SI. International funding for malaria control in relation to populations at risk of stable Plasmodium falciparum transmission. PLoS Med 2008; 5:e142.

Snow RW, Marsh K, (1995). Will reducing *Plasmodium falciparum* transmission alter malaria transmission among African children? *Parasitol Today* 11:188–190.

Stockholm Convention on Persistent Organic Pollutants, (2001). Convention Text. Geneva: United Nations Environment Programme. Available at http://www.pops.int/documents/ convtext/convtext_en.pdf

Takayama S, Sieber SM, Dalgard DW, Thorgeirsson UP, Adamson RH, (1999). Effects of long-term oral administration of DDT on nonhuman primates. *J Cancer Res Clin Oncol* 125: 219–25.

ter Kuile FO, Terlouw DJ, Kariuki SK, Phillips-Howard PA, Mirel LB, Hawley WA, Friedman JF, Shi YP, Kolczak MS, Lal AA, Vulule JM, Nahlen BL, (2003). Impact of permethrin treated bed nets on malaria, anemia, and growth in infants in an area of intense perennial malaria transmission in western Kenya. *Am J Trop Med Hyg 68 (Suppl 4):* 68–77.

ter Kuile FO, Terlouw DJ, Phillips-Howard PA, Hawley WA, Friedman JF, Kariuki SK, Shi YP, Kolczak MS, Lal AA, Vulule JM, Nahlen BL, (2003b). Reduction of malaria during pregnancy by permethrin-treated bed nets in pregnancy in an area of intense perennial malaria transmission in western Kenya. *Am J Trop Med Hyg 68 (Suppl 4):* 50–60.

Torres-Arreola L, Berkowitz G, Torres-Sánchez L, López-Cervantes M, Cebrián ME, Uribe M, López-Carrillo L. Preterm birth in relation to maternal organochlorine serum levels. *Ann Epidemiol* 2003; 13: 158–62.

Trape JF, Rogier C, (1996). Combating malaria morbidity and mortality by reducing transmission. *Parasitol Today* 12:236–240.

Turusov V, Rakitsky V, Tomatis L, (2002) Dichlorodiphenyltrichloroethane (DDT): ubiquity, persistence, and risks. *Environ Health Perspect* 110: 125–28.

USAID, 2007. Integrated Vector Management Programs for Malaria Vector Control. Programmatic environmental assessment. Available at: http://pdf.usaid.gov/pdf_docs/PNADI081.pdf

van den Berg H. Global status of DDT and its alternatives for use in vector control to prevent disease. Cien Saude Colet. 2011;16(2):575-90.

Van Wendel de Joode B, Wesseling C, Kromhout H, Moge P, Garcia M, Mergler D, (2001). Chronic nervous-system effects of long-term occupational exposure to DDT. *Lancet* 357:1014–1016.

Venners S, Korrick S, Xu X, Chen C, Guang W, Huang A, Altshul L, Perry M, Fu L, Wang Xet. Preconception serum DDT and pregnancy loss: a prospective study using a biomarker of pregnancy. Am J Epidemiol 2005; 162:709-716.

Vulule JM, Beach RF, Atieli FK, Mount DL, Roberts JM, Mwangi RW. Long-term use of permethrin impregnated nets does not increase Anopheles gambiae permethrin tolerance. Med Vet Entomol 1996;10:71-9.

Webb JL, Jr.: The first large-scale use of synthetic insecticide for malaria control in tropical Africa: lessons from Liberia, 1945-1962. J Hist Med Allied Sci 2011, 66:347-376.

Weill M, Lutfalla G, Mogensen K, Chandre F, Berthomieu A, et al. (2003) Insecticide resistance in mosquito vectors. Nature 423: 136–137.

WHO, (1973). Safe Use of Pesticides. WHO Technical Report Series No. 513. Geneva:World Health Organization.

WHO, (1979). World Health Organization. DDT and its derivatives. Environmental health criteria 9. Geneva: World Health Organization, United Nations Environment Programme, 1979.

WHO, (1999). World Health Organization. Safety of pyrethroid-treated mosquito nets. 1999. WHO Pesticide Evaluation Scheme (WHOPES). WHO Document: WHO/CDS/CPE/WHOPES/99.5. Available at: http://whqlibdoc.who.int/hq/1999/WHO_CDS_CPE_WHOPES_99.5.pdf

WHO, (2000a). The African summit on Roll Back Malaria, Abuja, 25 April 2000. Geneva: WHO. Publication number WHO/CDS/RBM/2000.17. Available: http://www.rbm.who.int/docs/abuja_declaration.pdf.

WHO, (2002). Final Report on the Third Meeting of the RBM Technical Resource Network on Epidemic Prevention and Control. Geneva: World Health Organization.

WHO, (2004). Frequently asked questions on DDT use for disease vector control.WHO/HTM/RBM/2004•54.Availableat:http://mosquito.who.int/ docs/FAQonDDT.pdf

WHO, (2005). Targeted subsidy strategies for national scaling-up for insecticide-treated netting programmes-principles and approaches. Geneva, World Health Organization, 2005. Available at: http://www.rbm.who.int/partnership/wg/wg_itn/docs/ts_strategies_en.pdf.

WHO, (2006a). World Health Organisation (2006) Pesticides and their application for the control of vectors and pests of public health importance. WHO/CDS/NTD/ WHOPES/GCDPP/2006.1.

WHO, (2006b). Indoor Residual Spraying: Use of Indoor Residual Spraying for Scaling Up Global Malaria Control and Elimination. Geneva: World Health Organization. Available at: http://whqlibdoc.who.int/hq/2006/WHO_HTM_MAL_2006.1112_eng.pdf

WHO, (2007). World Health Organization, 2007. WHO recommended insecticide products treatment of mosquito nets for malaria vector control, 2007. Available at: http://www.who.int/whopes/Insecticides_ITN_Malaria_ok3.pdf

WHO, (2008). World Health Organization (WHO), projections of mortality and burden of disease, 2004-2030, baseline scenario 2008. See: http://www.who.int/healthinfo/global_burden_disease/projections/en/index.html

WHO, (2009). World Health Organization, 2009. WHO recommended insecticides for indoor residual spraying against malaria vectors. Available at: http://www.who.int/whopes/Insecticides_IRS_Malaria_09.pdf

WHO, (2010a). World Malaria Report 2010. Executive summary and key points. Available at: http://www.who.int/malaria/world_malaria_report_2010/malaria2010_summary_keypoints_en.pdf

WHO, (2010b). World Malaria Report 2010. Operational coverage of insecticide treated nets, indoor residual spraying and antimalarial treatment, 2007–2009. Available at: http://www.who.int/malaria/world_malaria_report_2010/wmr2010_annex5.pdf

WHO, (2011). The use of DDT in malaria vector control: WHO position statement on DDT. Available at: http://whqlibdoc.who.int/hq/2011/WHO_HTM_GMP_2011_eng.pdf

Wolff MS, Zeleniuch-Jacquotte A, Dubin N, Toniolo P, (2000). Risk of breast cancer and organochlorine exposure. *Cancer Epidemiol Biomarkers Prev 9:* 271–277.

You L, Sar M, Bartolucci E, Ploch S, Whitt M. Induction of hepatic aromatase by p,p_-DDE in adult male rats. *Mol Cell Endocrinol* 2001; 178: 207–14.

Zaim M, 2002. Global Insecticide Use for Vector-Borne Disease Control. World Health Organization Pesticide Evaluation Scheme (WHOPES). Geneva: World Health Organization. Available at: http://whqlibdoc.who.int/hq/2002/WHO_CDS_WHOPES_GCDPP_2002.2.pdf

Zaim M, Guillet P, (2002). Alternative insecticides: an urgent need. *Trends Parasitol* 18: 161–163.

Zaim M, Jambulingam P, 2004. Global Insecticide Use for Vector-Borne Disease Control. World Health Organization Pesticide Evaluation Scheme (WHOPES). Geneva: World Health Organization. Available at: http://whqlibdoc.who.int/hq/2004/WHO_CDS_WHOPES_GCDPP_2004.9.pdf

Zaim M, Jambulingam P, 2007. Global Insecticide Use for Vector-Borne Disease Control. World Health Organization Pesticide Evaluation Scheme (WHOPES). Geneva: World Health Organization. Available at: http://whqlibdoc.who.int/hq/2007/WHO_CDS_NTD_WHOPES_GCDPP_2007.2_eng.pdf

Zaim,M.,Aitio, A.,Nakashima, N., 2000. Safety of pyrethroid-treated mosquito nets. Medical and Veterinary Entomology 14,1–5.