INSECTICIDES – PEST ENGINEERING

Edited by **Farzana Perveen**

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Preface

Agriculture is the mainstay of worldwide economy and the majority of urban and rural population of the world depends on it. Production of agricultural commodities is hindered by pest attacks. Sometimes the damage caused can be so severe that the economic yield of a crop is not possible. Insecticides are organic or inorganic chemical substances or mixtures of substances that can occur naturally or be synthesized, and are intended for preventing, destroying, repelling or mitigating the effect of any pest including avian, mammalian, crawling and flying insect pests.

Pesticides are divided to insecticides, fungicides, herbicides, rodenticides, acaricides and nematocides according to the organisms that they affect. There are various forms of insecticides; most are repellants or insect growth regulators used in agriculture, public health, horticulture or food storage. It is evident that insecticides have been used to boost food production to a considerable extent and to control disease vectors. Insecticides are used in various forms; from hydrocarbon oils, arsenical compounds, organochlorine, organophosphorus, carbamates, dinitrophenols, organic thiocynates, sulfur, sodium fluoride, pyrethroids and rotenone, to nicotine and bioactive natural products in solid or liquid form. These insecticides are highly toxic to pests and many others are relatively harmless to other organisms. Pests can respond to insecticides in at least two different ways: behavioral action, namely avoidance and toxicity.

A bacterium Bti is applied successfully in biological control programs against mosquitoes and flies larvae all over the world. The study of each of its facets is addressed in this book and will open new perspectives to improve their effectiveness in biological control.

Vector-borne diseases are a major contributor to the overall burden of diseases, particularly in tropical and sub-tropical areas, and a significant impediment to socio-economic development in developing countries. Insecticides still provide the most promising countermeasures to control malaria, dengue hemorrhagic fever (DHF) and other arthropod-borne diseases. The knowledge about the mosquito's behavioral responses to particular chemicals is very important for the prioritization and design of appropriate vector prevention and control strategies. Today, the development of insecticide resistance in insect pests and disease vectors occurs worldwide and on an increasing scale. This phenomenon suggests that behavioral responses will likely play a significant role in how certain pesticides perform to interrupt human-vector contact

while also reducing the selection pressure on target insects for developing resistance. 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. The relative resistance of mammals to pyrethroids is almost entirely attributable to their ability to hydrolyze the pyrethroids rapidly to their inactive acid and alcohol components, following direct injection into the mammalian CNS.

This book provides information on various aspects of pests, vectors, pesticides, biological control and resistance.

Farzana Perveen
Chairperson, Department of Zoology
Hazara University, Garden Campus
Mansehra
Pakistan

Part 1

Insecticides Mode of Action

1

Insecticide

A. C. Achudume
Institute of Ecology and Environmental Studies
Obafemi Awolowo UniversitY, Ile-Ife,
Nigeria

1. Introduction

Insecticides are organic or inorganic chemicals substances or mixture of substances intended for preventing, destroying, repelling or mitigating the effect of any insect including crawling and flying insects which may occur naturally or is synthesized (pyrethroids) e.g. organic perfumed and hydrocarbon oil and pyrethrins. There are various forms of insecticides. Most of the synthesized insecticides are by their nature are hazardous on health under the condition in which it is used. Insecticides therefore, range from the extremely hazardous to those unlikely to produce any acute hazard. Most are repellants and or insect growth regulators used in agriculture, public health, horticulture, food storage or other chemical substances used for similar purpose.

It is evident that insecticides have been used to boost food production to a considerable extent and to control vectors of disease. However, these advantages that are of great economic benefits sometimes come with disadvantages when subjected to critical environmental and human health considerations. Many insecticides are newly synthesized whose health and environmental implications are unknown.

Insecticides have been used in various forms from hydrocarbon oils (tar oils), arsenical compounds, organochlorine, organ phosphorous compounds carbonates, dinitrophenols, organic thiocynates, sulfur, sodium fluoride, pyethroids ,rotenone to nicotine, in solid or liquid preparation. Interestingly, most of these have been withdrawn due to the deleterious effect of the substances. Analysis of these formulations, their by- products and residues had in the past aided objective re–evaluation and re-assessment of these substances on a benefit–risk analysis basis and their subsequent withdrawal from use when found to be dangerous to human health and the environment. The quality and sophistication of these analyses have grown and very minute quantities of these insecticides or their residues can be analysed these days with a high degree of specify, precision and accuracy.

1.1 Inorganic and organ metal insecticides

The sequential organomentals and organometalloids insecticides are described in connection with the corresponding inorganic compounds. The highly toxic and recalcitrant compounds e.g. trichloro-bis-chlorophenyl ethane (DDT) and bis-chlorophenyl aqcetic acid (DDA) are formed unintentionally. The organic combination usually changes the absorption and distribution of a toxic metal and thus changes the emphasis of its effects, while the basic mode of action remains the same. The toxic effects of insecticides depend on the elements

that characterize it as inorganic or organmetal insecticides and on the specific properties of one form of the element or one of its components or merely on an inordinately high dosage. Some highly toxic elements such as iron, selenium, arsenic and fluorine are essential to normal development. The organometals and organometalloids are here described in connection with the corresponding inorganic compounds. Organic combination usually changes the absorption and distribution of a toxic metal and as a result changes the emphasis of its effects, but the basic mode of action remains the same.
The distinction between synthetic compounds and those of natural origin somewhat artificial. In practice, related compounds are assigned to one category or the other, depending on whether the particular compound of the group that was first known and used was of synthetic or of natural origin. For example, pyrethrum and later the naturally occurring pyrethrins were well known for years before the first synthetic parathyroid was made; as such, pyrethroid are thought of as variants of natural compounds, even though they have not been found in nature and are unlikely to occur.

1.2 Pyrethrum and related compounds

The insecticidal properties of pyrethrum flowers (chrysanthemum cinerarae- folum) have been recognized as insect powder since the middle of last century (McLaughlin 1973). In addition to their insect-killing activity, an attractive feature of the natural pyrethrins (pyrethrum) as insecticides was their lack of persistence in the environment and their rapid action whereby flying insects very quickly become incapacited and unable to fly. Prior the development of DDT, pyrethrum was a major insecticides for both domestic and agricultural use, despite its poor light stability. Development of synthetic pyrethroid with increased light stability and insecticidal activity allows it to be used as foliar insecticide while the natural pyrethrins are now used mainly as domestic insecticides.(Elliot, 1979).

1.3 Mode of Action

Pyrethrum and the synthetic pyrethroids are sodium channel toxins which, because of their remarkable potency and selection have found application in general pharmacology as well as toxicology (Lazdunski et al., 1985). Pyrethroids have a very high affinity for membrane sodium channels with dissociation constants of the order of $4x10^{-8}$ M (Sodeland,1985), and produce subtle changes in their function. By contrast, inexcitable cells are little affected by pyrethroids. The pyrethroids are thus referred to as open channel blockers.

1.4 Metabolism

The relative resistance of mammals to the pyrethriods is almost wholly attributable to their ability to hydrolyze the pyrethroids rapidly to their inactive acid and alcohol components, since direct injection into the mammalian CNS leads to susceptibility similar to that seen in insects (Lawrence and Casida, 1982). Metabolic disposal of the pyrethroids is very rapid (Gray et al., 1980), which means that toxicity is high by intravenous route, moderate by slower oral absorption, and often immeasurably moderate by slower oral absorption.

1.5 Poisoning syndromes

The pyrethroids are essentially functional toxins, producing their harmful effects largely secondarily, as a consequence of neuronal hyperexitability (Parker et al.1985). Despite this dependence on a relatively well-defined mode of action, the pyrethroids are capable of

generating a bewildering variety of effects in mammals and insects, which although showing some analogies with those produced by other sodium channel toxins (Gray, 1985; Lazdunski et al., 1985) and with DDT (Narahashi, 1986), have many unique characteristics (Ray, 1982b). Thus, toxicity of pyrethroids is divided into two groups Table 1. Type 1 pyrethroids produce the simplest poisoning syndrome and produce sodium tail currents with relatively short time constants (Wright et al., 1988). Poisoning closely resembles that produced by DDT involving a progressive development of fine whole-body tremor, exaggerated startle response, uncoordinated twitching of the dorsal muscles, hyperexcitability, and death (Ray, 1982b). The tremor is associated with a large increase in metabolic rate and leads to hyperthermia which, with metabolic exhaustion, is the usual cause of death. Respiration and blood pressure are well sustained, but plasma noradrenalin, lactate, and adrenaline are greatly increased (Cremer and Servile 1982). Type 1 effects are generated largely by action on the central nervous system, as shown by the good correlation between brain levels of cismethrin and tremor (White et al., 1976). In addition to these central effects, there is evidence for repetitive firing in sensory nerves (Staatz-Benson and Hosko, 1986).

Type I	**Intermediate**	**Type II**
Allethrin	Cyhenothrin	Cyfluthrin
Barthrin	Fenproponate	Cyhalothrin
Bioalethrin	Flucythrinate	Cypermethrin
Cismethrin		Deltamethrin
Fenfluthrin		Fenvalerate
Trans-fluorocyphenothrin		Cis-fluorocyphenothrin
Kadethrin		
Permethrin		
Phenothrin		
Pyrethrin I		
Pyrethrin II		
Resmethrin		
Tetramethrin		

Table 1. I Acute toxicity of pyrethroids (Wright et al., 1988; Forshow and Ray, 1990).

The type 11 pyrethroid produces a more complex poisoning syndrome and act on a wider range of tissues. They give sodium tail currents with relative longterm constants (Wright, et al., 1988). At lower doses more suble repetitive behavior is seen (Brodie and Aldridge, 1982). As with type I pyrethroids, the primary action is on the central nervous system, since symptoms correlate well with brain concentrations (Rickard and Brodie, 1985). As might be expected, both classes of parathyroid produce large increases in brain glucose utilization (Cremer et al. 1983). A final factor distinguishing type 11 pyrethroids is their ability to depress resting chloride conductance, thereby amplifying any sodium or calcium effects (Forshaw and Ray, 1990).

Intermediate signs representing a combination of type I and type 11 are produced by some pyrethroids. These appear to represent a true combination of the type I and 11 classes (Wright et al., 1983) and thus represent a transitional group. Evidence in support of this is given by measurement of the time constants of the sodium after potential produced by the

pyrethroids. Since pyrethroids appear to be essentially functional toxins, they produce few if any specific neuropathological effects.

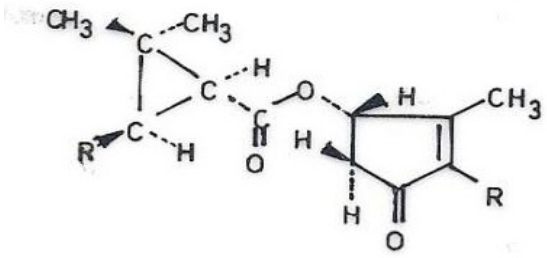

Fig. 1. Pyrethrins of the form.

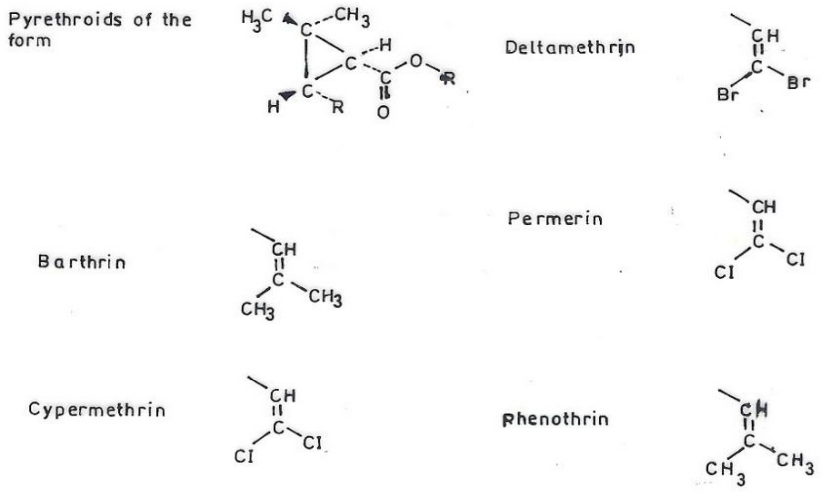

Fig. 2. Pyrethroids of the form.

1.6 Identity, properties and uses

The six known insecticidal active compounds in pyrethrum are esters of two acids and three alcohols. Insect powder made from "Dalmatian insect flower" (*Chrysanthemum cinerariaefolium*) is called pyrethrum powder or simply pyrethrum. The powder itself was formerly used as an insecticide, but now it is usually extracted. The six active ingredients are:

- Pyrethrin I – pyrethrolone ester of chrysanthemic acid
- Pyrethrin II- pyrethrolone ester of pyrethric acid
- Cinerin I- cinerolone ester of chrysanthemic acid
- Cinerin II- cinerolone ester of pyrethric acid
- Jasmolin I- jasmolone ester of chrysanthemic acid
- Jasmolin II- jasmolone ester of pyrethric acid

The six known insecticidally active compounds in pyrethrum are esters of two acids and three alcohols. Specifically, pyrethrins l is the pyrethrolone ester of chrysanthermic acid, -pyrethrin II is the pyrethrolone ester of pyrethria acid, cinerin l is the cinerolone ester of chrysanthemic acid, cinerin II is the cinerolone ester of pyrethric acid, jasmolin I is the jasmolone ester of chrysanthemic acid and jasmolin II is the jasmolone ester of pyrethric acid. There is much evidence indicating that the biological activity of these molecules depends on their configuration (Elliot, 1969, 1971).
The six active ingredients are known collectively as pyrethrins; those based on chrysanthemic acid are called pyrethrin I, and those based on pyrethric acid are called pyrethrins II. Pyrethrins, generally combined with a synergist, are used in sprays and aerosols against a wide range of flying and crawling insects. Usually about 0.5% active pyrethrum principles are formulated. They are equally effective for control of head lice and flea in dogs and cats.

1.7 Raid as insecticide

The insecticide 'Raid' belongs to a group of chemically stable pyrethrin, has widespread use in control of insects. Chemical stability, insecticide and organic phosphorus hydrocarbon have been shown to accumulate rapidly in tissues causing death and have profound effect on growth (Nebeker et al., 1994). Insecticide raid shows no observable effects on mortality and growth at lower test concentrations in rats. At higher concentration of 430 and 961 µg/g, survival decreased as concentration increases. In addition, mean total body weight of animals fed insecticides raid with concentrations of 430 and 961 µg/g were significantly decreased ($P<0.05$) than the controls. Conclusively, the higher the concentration of the insecticide Raid, the more hazardous it has on cell death (Achudume et al., 2008)(Table II).
Bioaccumulation factor of insecticide Raid was observed in lipids, up to three times that of the feed at the first concentration and gradually decreases as the concentrations increase (Table III), whereas accumulation factor in the muscle (0.7), brain (0.5), and liver (0.3) was about the indicated number times that of the feed. At higher concentration of 961 µg/g, bioaccumulation factor decreased in the lipid to 1.2 and 0.6 in the muscle, 0.03 in the brain, and 0.08 in the liver. Using the mean of insecticide in feed, the tissues accumulate the insecticide in the following ascending order: brain < liver < muscle < lipid. Similarly, Table III indicates the estimated detectable levels of toxicity in rat tissues exposed to the insecticide Raid. The brain shows mild decrease in toxicity of the enzymes glucose-6-phosphatase and lactic acid dehydrogenase, whereas significant decreases were noticeable in the muscle and liver (Achudume et al. 2008).
Long-term exposure of insecticide had been reported to result in systemic toxicity such that may impair the function of the nervous system and increase the risk of acute leukemia in children (Menegaux et al., 2006). Also, pesticides including organ phosphorus insecticides used against crawling and flying insects in homes have the potential of being carcinogens (Peter and Cherion, 2000). The adverse effect of insecticide Raid was demonstrated in a study by increase in alkaline phosphates activity in both plasma and liver which is a known measure of hepatic toxicity, and confirms "Raid" as a hepatotoxicant. The significant increase in alkaline phosphates activity (Table IV) may be due to hepatocellular necrosis which causes increase in permeability of cell membrane resulting in the release of this enzyme into the blood stream. The insecticide Raid significantly decreased reduced glutathione levels especially in the liver and this has implications for the ability of the animal to withstand oxidative stress. Studies have shown that GSH deficiency in cells is

associated with markedly decreased survival (Kohlmeier et al., 1997), thus, chemically stable lipid-soluble, organophosphorus insecticides are hazarddous to health through mechanisms including depletion of GSH (Menegaux et al., 2006).

Means SD concentrations of insecticide "Raid" in feed (Mg/g)	Mortality	Means: SD body weight (g)
0.00	Nil	135=5.4
25.0=2.4	Nil	135=21.7
54.0=9.5	Nil	132=2.9
108.2=12.5	Nil	129=3.2
216.2=14.6	Nil	128=19.8
430.0=20.2	1	118=20.5
961.2=70.5	2	116=5.3

Table 1. II mortality and growth of wistar rats exposed to different concentrations of "Raid".

Mean±SD Insecticide "Raid" in Feed (µg/g)	Raid Concentration in Wistar Rats (µg/g)[a] and Bioaccumulation Factor (BAF) Lipid	Muscle	Brain	Liver
00.0	-	-	-	-
25.0±2.4	72.5± (2.9)	17.5(0.7)	12.5(0.5)	7.5(0.3)
54.0±9.2	86.4(1.6)	21.7(0.4)	16.4(0.3)	9.4(0.2)
108.2±12.5	172.8(1.6)	30.4(0.3)	19.5(0.2)	10.8(0.10)
216.2±14.6	280.8(1.3)	45.8(0.2)	22.9(0.1)	19.8(0.09)
430.0±20.6	324.0(0.8)	86.4(0.2)	25.8(0.06)	37.3(0.09)
961.2±70.5	1153.2(1.2)	576.6(0.6)	28.8(0.03)	76.9(0.08)

Table 1. III Tissue total raid concentrations and bioaccumulation factors (BAF) in wistar rats.

Raid concentrations Tissue In feed (µg/g)		Alk pase activity µgml^{-min-L}	GSH level mg/ml	Glucose level mg/g liver
430±20.2	Control	0.08±0.04	0.18±0.02	0.96±0.04
	Plasma	0.06±0.09	0.15±0.6	0.90±0.04
	Control	0.08±0.04	0.18±0.02	0.94±0.01
	Liver	0.06±0.02*	0.15±0.01	1.05±0.12
961.2±70.5	Control	0.09±0.05	0.19±0.05	0.96±0.52
	Plasma	0.06±0.01	0.11±0.05	1.09±0.52
	Control	0.08±0.08	0.19±0.02	0.96±0.06
	Liver	0.05±0.08*	0.09±0.03*	1.66±0.04

Data values are mean±SD
*Statistically significant p< 0.05

Table 1. IV Effect of Raid concentrations in feed on hepatic enzyme activity, reduced glutathione and glucose levels.

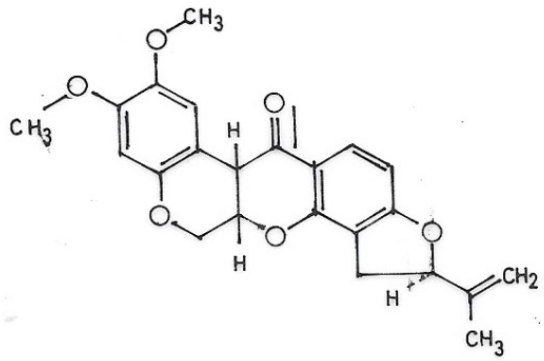

Fig. 3. Structure of rotenone.

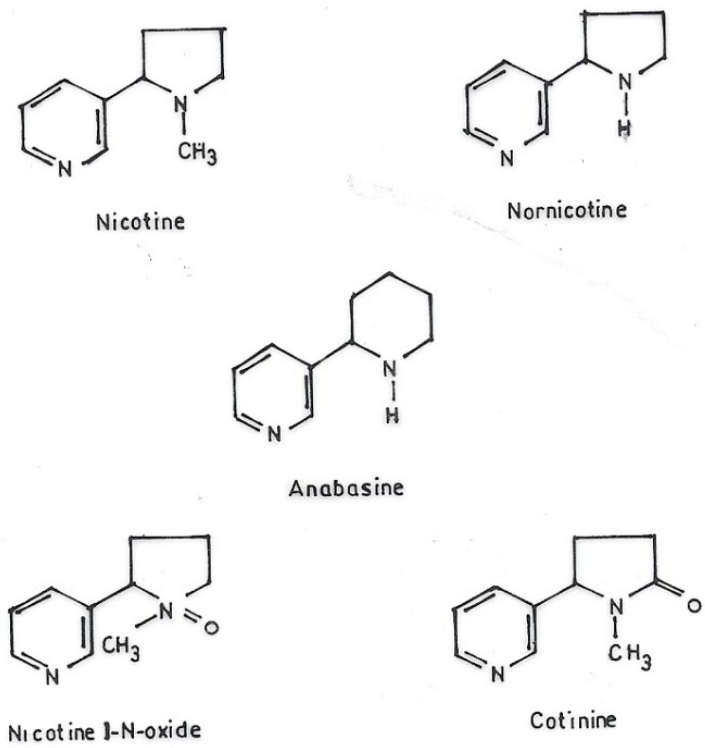

Fig. 4. Nicotine, narnicotine and anabasine with two important metabolites of nicotine.

Some other studies confirm that glutathione deficiency is associated with impaired survival in HIV disease (Herzenbery et al., 1997). Glutathione may be consumed by conjugation reaction, which mainly involve metabolism of zenobiotic agent. However, the principle mechanisms of hepatocyte glutathione turnover are known to be by cellular efflux (Sies et al., 1978). Glutathione reducatase is a known defense against oxidative stress, which in turn needs glutathione as co-factors. Catalase is an antioxidant enzyme which destroys H_2O_2 that can form a highly reactive radical in the presence of iron as catalyst (Gutter ridge, 1995).

Achudume et al., 2008 showed that bioaccumulation factor of insecticides raid was observed in lipid. Lipid peroxidation is a chemical mechanism capable of disrupting the structure and function of the biological membranes that occurs as a result of free radical attack on lipids. Some study confirms that insecticide raid increased lipid peroxidation, oxidative stress and hepatotoxicity due to reduced antioxidant system.

In addition, SOD is family of metalloid enzyme which is considered to be stress protein which decreases in response to oxidative stress (McCord, 1990). It is evident that decrease of SOD in the tissue is a confirmation of its protection from damage caused by insecticide Raid.

2. Classes of insecticides

- The classification of insecticides is done in several different ways (Hayes, 1982),(Heam 1973, Lehman, 1954, Martin and 1977).

- Systemic insecticides are incorporated by treated plants. Insect ingest the insecticide while feeding on the plants.
- Contact insecticides are toxic to insects by direct contact. Efficacy is often related to the quality of pesticide application in aerosols which often improve performance.
- Natural insecticides, such as pincotine, pyrethrum and neem extracts are from plants as defences against insects.
- Inorganic insecticides are manufactured with metals e.g. Heavy metals
- Organic insecticides are synthetic chemicals which comprise the largest numbers of pesticides available.

Insecticides are pesticides used to control insects many of these insecticides are very toxic to insects and many others are relatively harmless to other organism except fish.
Insecticides decompose readily so the residues do not accumulate on crops or in the soil. Insecticides include ovicides and larvicides used against the eggs and larvae of insects respectively.
The use of insecticides is believed to be one of the major factors behind the increase in agricultural productivity (McLaughlin,1973, van Emden and Pealall, 1996). Nearly all insecticides have the potential to significantly alter ecosystems; many are toxic to humans; and others are concentrated in the food chain (WHO 1962, 1972). Selected inorganic metals are discussed in the next section followed by individual insecticides organ metals.

2.1 Barium

Barium is an alkaline earth metal in the same group as magnesium, calcium, strontium and radium. It valence is two. All are water-and acid soluble compounds. They are poisonous. Barium carbonate is a rat poison. It is used in ceramics, paints, enamels, rubber and certain plastics.
Absorption, Distribution, Metabolism Excretion (ADME): Barium carbonate is highly insoluble in water. It is partially solubilized by acid in the stomach. The danger of the insecticide is through ingestion. Various barium compounds can cause pneumoconiosis. It is absorbed from gastrointestinal tracts of rat rapidly and completely. It is stored in bone and in other tissues (Hayes 1982, Castagnou et al 1957, Dencker et al., 1976, 83). Excretion takes place rapidly in urine and feces in 24hr (Bauer et al. 1956).
Mode of action: Barium stimulates striated cardiac and smooth muscle, regardless of the innervation.

2.2 Chromium

Chromium is a metal somewhat like iron and separated in the periodic table by manganese. Only hexavalent chromium compounds (chromates) are important as pesticides. They are also the most toxic. Chromate is absorbed by the lung (Baetjer et al., 1959), gastrointestinal tract and skin. It is widely distributed in the liver, kidney, bone and spleen (Mackenzie et al 1958). Acute poisoning may produce death rapidly through shock or renal tubular damage and uremia (Steffee and Baetjer 1965).

2.3 Mercury

Mercury is toxic no matter what its chemical combination. It is widely distributed in the environment, and traces of it occur in food, water and tissues even in the absence of occupational exposure. Inhaled mercury vapour diffuses across the alveolar regions of the

lung into the blood stream. Mercury vapour is a monatomic gas which is highly diffusible and lipid soluble (Berlin et al 1969a , Hush,1985). Once in the bloodstream mercury vapour enters the blood cells where it is oxidized to divalent inorganic mercury under the influence of catalase (Halbach and Clarkson 1978). Mercury is widely distributed with the highest concentrations in the kidney.

2.4 Thallium

Thallium stands between mercury and lead in the periodic table, and compounds of these metals show marked similarities. All of them may produce immediate local irritation followed by delayed effects in various organs, notable the nervous system. Thallium sulphate has been more widely used as pesticide than any other compound of thallium. It has produced many cases of poisoning and serves as good example of the toxicity of thallium generally (Lund, 1956b).

Thallium is easily absorbed by the skin as well as by the respiratory and the gastrointestinal tracts. Thallium accumulates in hair follicles and much less in those in the resting phase. Excretion is slow and is entirely by urine in humans but in rats via faeces (Barclay et al., 1953, Lund, 1956a)

2.5 Lead arsenate

Lead arsenate includes acid lead arsenate, dibasic lead arsenate, dilead orthoarsenate, diplumbic hydrogen arsenate, lead hydrogen arsenate and standard lead arsenate. Lead arsenate is used as an insecticide. it is used to control moths, leaf rollers and other chewing insects and in soil for the treatment of Japanese and Asian beetles in lawn. Absorption is generally via gastrointestinal. Dermal absorption is extremely small. Lead and arsenate are distributed separately in the body. lead is stored in highest concentration in the bone with much lower concentrations in soft tissues. Arsenic is stored in the liver and in some instances in the kidney at higher concentrations than those for lead (Fairhall and Miller, 1941. Lead is transferred to the fetus of animals humans (Heriuchi et al., 1959).

2.6 Antimony potassium tartrate

This compound serves as a poison in baits to control insects, especially thrips, and as an emetic in bait to control rodents. Ingestion of the compound usually leads to repeated vomiting. Excretion is mainly urinary (Fairhall and Hyslop, 1947).

2.7 Sodium selenate

Sodium selenate is an insecticide used in horticulture for control of mites, aphids and mealybugs. Various compounds of selenium are freely absorbed from the respiratory and gastrointestinal tracts. Dermal absorption is less important. Selenium is stored more in the liver, kidney, spleen, pancreas, heart and lung than in other organs (Underwood, 1977). Selenium is excreted chiefly in the urine but about 3-10% is metabolized and excreted by the lungs and through faecal excretion.

2.8 Sodium fluoride

Sodium fluoride is toxic to all forms of life. It has been used as an insecticide, rodenticide and herbicide and as fungicide for preservation of timber. Its toxicity to plants generally has

restricted its use as an insecticide to bait formulations (who, 1970). Sodium fluoride concentrates more in the plasma and liver and is excreted in urine.

3. Miscellaneous elements

3.1 Boric acid

Boric acid and borax have been used as an insecticide, both mainly for the control of cockroaches. Boric acid also is known as boracic acid and as orthoboric acid. Absorption from the gastrointestinal tract is rapid and virtually complete. Its peak concentration is in brain and less in other tissues. Boric acid is excreted unchanged in the urine (wong et al., 1964).

3.2 Insecticides derived from living organisms and other sources:

Different groups of insecticide; derived from living organisms are entirely unrelated chemically and pharmacologically. They range from relatively simple alkaloids such as nicotine, with a molecular weight of only 162.2, through proteinaceous poisons to virulent living organism. They range in toxicity from harmless and fragile pheromones, which are used as a chemical warfare agent.

The distinction between synthetic compounds and those derived from living organisms is somewhat artificial. In practice, related compounds are assigned to one category or the other, depending on whether the particular compound of the group that was first known and used was of synthetic or of natural origin. For example, pyrethrum and later the naturally occurring pyrethriums were well known for years before the first synthetic pyrethroid was made; as a result, pyrethroids are thought of as various of natural compounds, even though they have not been found in nature and are unlikely to occur. By contrast, synthetic sodium fluoroacetate acquired a reputation as a rodenticids and was explored as a synthetic insecticide before it was realized that the potassium salt is the active principal of a poisonous plant. Thus pyrethroids are discussed extensively.

Perhaps the only unifying feature of the diverse array of poisons derived from living organism is the popular view that "natural" substances are harmless. On this matter of safety, an expert committee of the world health organisation pointed out that "all the most poisonous materials so far know are, in fact, of natural origin" (WHO,1967).

3.3 Pyrethrum and related compounds

The insecticidal properties of pyrethrum flowers (genus chrysanthemum) have been recognized since the middle of 1st century, when commercial sale of "insect powder" from Dalmatian pyrethrum flower heads began (McLaughlin, 1973). In addition to their insect-killing activity, their lack of persistence in the environment and rapid "knock down" activity whereby flying insects become uncoordinated and unable to fly makes it very useful. Pyrethrum used to be a major insecticide for both domestic and agricultural use despite its poor light stability. Its usefulness was extended by introduction of piperonyl butoxide and other compounds as synergists, which greatly reduced the unit cost of crop treatment. Development of synthetic pyrethroids with increased stability and insecticidal activity (Elliot 1977) reduced the use of pyrethrum. However, natural pyrethrins are now used mainly as domestic insecticides, while the synthetic pyrethroids represented 20-25% of the world foliar insecticide market in 1983 (Herve's 85) and the proportion is increasing

steadily. Thousands of new synthetic pyrethroids have been synthesized, some showing complete divergence from the original pyrethrins (casida et al., 1973). Table 2.1 and Table 2.2.

4. Mode of action

Pyrethrum and the synthetic pyrethroid are sodium channel toxins which, because of their remarkable potency and selectivity, have found application in general toxicology (Lazdunski et al 1985). Their actions on the nerve membrane sodium channel are well understood. Pyrethroids have a very high affinity for membrane sodium channel, they have little effect on inactive sodium channels or close channels and produce subtle changes in their functions. After modification by prethroids, sodium channels continue in many of their normal functions, retaining their selectivity for sodium ions and link with membrane potential (Narahashi, 1986). The pyrethroids are thus known as open channel blockers. Detailed studies can be found in Narahishi 1986, Jacques et al., 1980, and Gray 1985.

4.1 Metabolism

The relative resistance of mammals to the pyrethroids is almost wholly attributable to their ability to hydrolyze the pyrethroids rapidly to their inactive acid and alcohol components, since direct injection into the mammalian CNS leads to susceptibility similar to that observed in insects (Lawrence and Casida, 1982). Some additional resistance of homoeothermic organisms can be attributed to the negative temperature coefficient of action of the pyrethroids (Van den Bercken et al., 1973) which are thus less toxic at mammalian body temperature but the major effect is metabolic.

The metabolic pathways for the breakdown of the pyrethroids vary little between mammalian species but vary somewhat with structure. This literature has been ably summarized by Leahy (1985), and further references to the metabolism of specific pyrethroids are given in the sections on individual compounds. Generally pyrethrum and allethrin are broken down mainly by oxidation, whereas for the other pyrethroids ester hydrolysis predominates. These reactions can take place in both liver and plasma and are followed by hydroxylation and conjugation to glucuronides or sulphates, which are then excreted in the urine (Gray 1985).

4.2 Individual insecticides

Other known insecticides pyrethroids under organophosphates are listed below only selective ones are discussed.

Allethrin	Permethrin
Bifenthrin	
Cyhalothrin, Lambda-cyhalothrin	
Cypermethrin	Phenothrin
Cyfluthrin	Prallethrin
Deltamethrin	Resmethrin
Ftofenprox	Tetramethrin
Fenvalerate	Transfluthrin

Table 4. I Other known insecticides.

4.3 Cypermethrin

Cypermethrin (R, S)-∞- cyano-3-pheno-xybenzyl-2, 2-dimethyl. There are eight isomeric forms. It was introduced commercially in 1977 as an emulsifiable concentrate to be used against a wide range of insect pest (Elliot, 1977).

4.4 Deltamethrin

Deltamethrin S-∞- cyano-3-phenoxybenzyl-(IR)-cis-3-(2, 2-dibromovinyl)2,2-dimethcyclo-propane carboxylate. It is a single isomer. It is used against a wide range of insect pests. It produces a typical type II motor symptom in mammals (Barnes and verschoyle, 1974. Metabolism of deltamethrin involves rapid ester cleavages and hydroxylation (Shono eta al; 1977).

4.5 Fenproponate

Fenproponate(∞-cyano-3-phenoxybenzyl-2,2,3,3-tetra-methylcyclopropanecarboxylate).
There are eight isomerism forms. Fenpropathrin is another common name, was first developed by sumitomo and commercialized in 1980 as an emulsifiable concentrate to be used against a wide range of insect pests. Fenproponate produces intermediate or mixed motor symptoms in mammals (Wright et al., 1988).

4.6 Fenvalerate

Fenvalerate (R,S)- ∞- cyano-3-phenoxy-benzyl (IR,IS)-2-(4-chlorophenyl)-3-methyl-1-butyrate. There are four isomeric forms. It should be noted that fenvalerate is not based on a cyclopropane ring structure. It was introduced commercially to be used against a wide range of insect pests fenvalrate produces typical type II motor symptoms in mammals (Verschoyle and Aldrige, 1980).

4.7 Phenothrin

Phenothrin (3-phenoxybenzy-(IR,IS)-cis,trans-3(2-methylprop-1-enyl)-2,2 dimethyicyclo-propane carboxylate). There are four isomeric forms. It is used as a domestic insecticide in a partially resolved mixture rich in the IR isomer (Sumithrin) and for grain protection. Phenothrin produces typical type 1 moto symptoms in mammals (Lawrence and Casida, 1982)

4.8 Rotenone and related materials

Rotenone-bearing plants have longed being used as a fish poison by many ancient different indigenous people, nut their use as an insecticide is probably more than a century old. Plants known to produce rotenone and other rotenoids belong to at least 68 species of the family Leguminosae, the same as that for peas and beans. The genera most exploited so far are Derris, native to southeast Asia, and lonchocarpus to south America (shepard 1951).
Rotenone and other active principles often occur chiefly in the roots of rotenone bearing plants but may be in the leaves (as in *Tephrosia vogeli*), seeds (as in *Milletia pachycarpa*), or bark (as in *Mundulea serica*).
Regardless of the genus or the particular part of the plant involved, the active constituents of rotenone-bearing plants may be extracted with ether or acetone as resin.
Rotenone is (2R,6a 5,12a 5)-1,2,6,6a,12,12a-hexahydro-2-isopropenyl-8,9-dimethoxy-chromeno (3,4-b) furo(2,3-h) chromen-6-one. Its structure is depicted in fig. 3. Although

rotenone generally is considered to be the active ingredient in all resins isolated, the other constituents show considerable insecticidal activity (Metcalf, 1955).
Rotenone is readily oxidized and racemized in the presence of light and the process is accelerated in alkaline solution (Cheng et al., 1972). It is active as a nonsytemic pesticide against a wide variety of insects, arachnids and molluscs. Its rapid photodecomposition means that it is active only for about 1 week on plants or 2-6 days in water and this limits its commercial use though still finds use as a domestic garden insecticide.
Rotenone is a highly potent mitochondrial poison, blocking NADH oxidation, this property dominates its actions in animals (Heikkila et al., 1985).
Rotenone is metabolized rather effectively by the liver in isolated rat liver mitochondria, the aerobic oxidation of pyruvate is almost completely inhibited by rotenone (Haley 1978).

4.9 Nicotine and related compounds

Three closely related compounds (nicotine, nornicotine and anabasine fig4) were commonly used as insecticides, although only the most potent, nicotine, is now used to any extent. Nicotine is usually obtained from the dried leaves of nicotiana tabacum, but it also occurs in *N. rustica* and *Duboisia*, another genus of the solanaceae, and in three other taxonomically diverse general, namely Asclepia (Asclepidaceae), Equisetaceae (Equisetaceae), and Lycopodium (Lycopodiaceae); Nicotine (S-3-(1-methyl pyrrolidin-2-yl) pyridine) is used as nicotine sulphate as a stomach poison for leaf eating insects (Haigh and Haigh 1980). Nicotine is rapidly absorbed from all mucosal surfaces, including those of the mouth, gastro-intestinal tract, and lung. Since nicotine readily forms salts in acid solution, its penetration through biological membranes is strongly pH dependent (Schievelbein, 1982).
The metabolism of nicotine is highly complex and reviewed by Gorrod and Jenner (1975) and schievelbein (1982). Metabolism mainly by cytochrome P.450 linked microsomal oxidative pathways in the liver. Cotinine (Fig4) is major metabolite, which then undergoes further oxidation. Nicotine stimulates the action of acetylcholine at nicotinic receptors in the central nervous system, autonomics ganglia and some pheripheral nerves. It central actions result in tremor and convulsions, stimulation and then depression of ventilation and induction of vomiting by a direct action on the medulla. Ventilation is stimulated by peripheral actions on the aortic and carotid chemoreceptors, and adrenal catecholamine. Secretion is increased at low doses. Heart rate and blood pressure are largely dominated by sympathetic effects and show increases compounded by adrenal catecholamines. The gastrointestinal tract is dominated by parasympathetic effects and shows hypersecretion followed by block as well as increased tone and peristalsis. Death is usually a result of block of neuromuscular transmission in the respiratory muscles or a consequence of seizures. In addition to its action on cholinergic transmission, nicotine can act at noncholinergic sites and also activate receptors on sensory nerve endings and vagal C fibers (Martin, 1986).
The carcinogenic potential of tobacco is well established, but there is debate about the role of nicotine, which, although probably not carcinogenic itself can be converted to carcinogens such as N'-nitrosonornicotine and 4-(methyl-nitrosamino)-1-(3-pyriyl)-1-butanone. The metabolites cotinine and nicotine 1-N-oxide are not carcinogenic although they do produce hyperplasia of the bladder epithelium (Hoffmann et al., 1985).

5. Living organisms as pesticides

The use of biological control agents has many potential advantages over chemical control, not least the possibility of high selectivity for the predators and other beneficial species. Several microorganisms or microbial products have been identified as potential insecticides (Miller et al, 1983). Most successful attempts have been directed against insects, as biological control of vertebrates has met with little success due to cross-infection problems. The world Health Organisation has investigated viruses, bacteria fungi and nematodes as potential insect control agents since all play a part in limiting the growth of natural insect populations.

5.1 Viral insecticides

Viral insecticides are still in the experimental stage but many are under investigation, as reviewed by Miller et al., 1983. Bacterial insecticides represent the largest and widest used group and reviewed by Burges (1982) and Lysenko(1985). All of those used are spore-formers, since the spores can be readily stored in dried form and applied by conventional means as wettable powders or dusts. Many form a crystalline toxin within the spore which enhances their pathogenicity to insects. The most widely used is *Bacillus thuringiensis*. A closely similar bacterium, *Bacillus papilliae* has been used against Japanese beetle. It has the advantage that once spores are introduced into the environment the bacterial population is sustained by reinfection of the insect hosts, but the disadvantage that spore production requires expensive in-vivo production using insect pupae and is now of declining importance. It is highly specific, does not infect vertebrates, and despite production of a crystal toxin is nontoxic to mammals by repeated oral administration (Burges, 1982).

5.2 Fungal insecticides

Fungal insecticides are commercially produced for a variety of specific applications. Their importance in controlling natural insect populations has been recognized since 1834, Aschersonia has been used to control Floridian white fly on citrus since the early 1900s. Fungi have the advantages of forming a stable population in the insect environment and are capable of infection through the insect cuticle, not by ingestion as bacteria. A disadvantage is their susceptibility to widely used fungicides. Examples include *Beauveria basiana* is marketed as Boverin and used against Colorado beetle and corn borer in Russia and China. *Metarhizium anisopliae* was used against a range of insects as metaquino. *Hirsutella thompsoni*, is used to control citrus rust in the united states as myear and *Vecticillium lecani* is used as vertalec or mycotal for aphid control in united kingdom. Some fungi such as *Beauveria bassiana* produce toxins which may be involved in their pathogenicity. *Culicinomyces clavosporus* and *lagenidium giganteum* are mosquito pathogens (Miller et al. 1983).

5.3 Nematate insecticides

Nematate insecticides have been isolated from mosquito larvae at low natural population densities. They are reared in vivo, which is expensive, and there some resistant mosquito population Nametodes are tolerant of many insecticides and insect growth regulators and can be used in combined malaria control programs and are rapidly broken down by human gastric juice (Gajana et. al; 1978).

6. Conclusion

Given the enthusiasm of the proponents of biological insect control and the limited role that these agents play in current pest control may be perhaps surprising. There are however, a number of difficulties in sustaining a usefully large population of the control agent on crops, or in the case of mosquitoes at the water surface, and in agriculture difficulties associated with the very high host specificity of some agents. More fundamental problems are the potential risk from replicating agents which can increase in the environment and the possibility of transfer of toxin encoding genes from invertebrate to vertebrate bacteria or viruses. It is clear, however, that current experience with biological control agents is very encouraging and that they can be expected to play an important part in integrated pest control programs in the future (Laird, 1985).

While animals as well as humans may be adversely affected mainly by ingestion of the active ingredients, the effect of propellant chemical cannot be ignored. Inflammatory activation might be an important mechanism underlying toxicity effects in the tissue (Mense et al., 2006). The role of propellant in the toxicity of insecticide Raid may not be cleared. A comprehensive assessment of the risk associated with environmental use of insecticide Raid was determined in various tissues as it affects the basal biochemical molecules of cells (Achudme et al., 2008).

7. References

Achudume, A.C., Nwoha, P.C., Ibe, J.N. (2008) Toxicity and Bioaccumulation of insecticide "Raid" in Wistar Rats Inter Environ Toxicity 24(4); 357-361.

Barcley, R.K;peacock, W.C. and Karnofsky,G.A. (1953). Distribution and excretion of radioactive thallium in the chick embryo, rat and man.J. Phaemacol.Exp.Ther 107, 178-187.

Barnes, J.M. and Verschoyle, R.D. 1974 Toxicity of new Pyrethroid insecticide. Nature (London) 248-711.

Bauer, G.C.; Carlsson, A. and Lindquist, B. (1956). A comparative ^{45}Ca in rats. Biochem. J. 63, 535-542.

Berlin, M.H; Nordbery, G.F. and Serenius, FR. (1969a). on the site and mechanism of mercuric nitrate Hg. 203.Arch.Environ. Health 18, 42-50.

Brodie, M.E. and Aldridge, W.N 1982 Elevated cerebellar cyclic GMP levels during the deltamethrin induced motor syndrome. Neurobehav. Toxicol. Teratol 4, 109-113.

Burges, H.D. (1982). Control of insects by bacteria. Parasitology 84, (symp), 79-117.

Casida, J.E. (1973). Biochemistry of the pyrethrins. In "pyrethrum: The Natural insecticide" (j.E. casida, ed). Academic press, New York and London.

Castagnous, R; Paolett; C. And Larcebeau, S. 1957. Absorption and distribution of barium administered intravenously or orally to rats C.R. Hebd, seanes ser Acad Sci D 244, 2994-2996. (in French).

Cheng, P.Y., Buster, D., Hommock, B.D., Roe, R.M, and Alford, A.R. 1987. *Bacillus thuringiensis* van. Israelenisio-enctotosian Evidence of Neurotoxic action. Pestic Biochem Physiol. 27, 42-49.

Cremer, J.E and Seville, M.P. 1982-Comparative effects of two Pyrethroids, deltamethrin and cismethrin on plasma catecholamines and on blood glucose and lactate. Toxicol. Appl Pharmacol. 66, 124-133

Cremer, J.E., Cunningham, V.J. and Seville, M.P. 1983. Relationship between extraction and metabolism of glucose, blood flow, and tissue blood volume in regions of rat brain. J. Cereb. Blood Flow Metab. 3, 291-3002.

Dencker, L; Danielsson, B; khayal, A, and Lindren, A. (1983). Deposition of metals in the embryo and fetus. In "Reproductive and Developmental Toxicity of metals" (T.W. Clarkson, G.F Nordberg, and P.R. sage eds), pp 607-631. Plenum, New York.

Dencker, L; Nillson, A; Ronnback, C. And walinder, G. (1976). Nptake and retention of ^{133}Ba and ^{140}Ba-^{140}La in mouse tissue. Acta Radiol: Ther; Phys; Biol (N.S) 15, 273-28.

Elliot, M. 1977. "Synthetic Pyrethroids", ACS Symp. Ser. No. 42 Am. Chem. Soc., Washington, D.C.

Elliot, M., 1971. The relationship between the structure and the activity. Chem. Ind. (London) 24 776-791.

Fairhall, L.T. and Hyslop, F. (1947). The toxicology of antimony. Public Health Rep; suppl. 195.

Fairhall, L.T. and miller, J.W. (1941). A study of the relative toxicity of the molecular components of lead arsenate public Rep 56, 1610-1625.

Forshaw, P.J and Ray, D. E. 1990. A novel action of deltamethrin on membrane resistance in Mammalian skeletal muscle and non-myelinated nerve fibres. Neuropharmcol. 29, 75-81.

Gajana, A; kazimi, S.J; Bheemarao U.S., Suguna, S.G; and chandrahas, R.K 1978 studies on a nematode parasite (Romano-mermis sp; mermithidae) of mosquito larvae isolated in Pondicherry Indian J. Med Res. 68, 242-247.

Gorrod, J.W, and Jenner, P. 1975. The metabolism of tobacco alkaloids. Essays Toxical 6,35-78 schievelbein, H 1982.

Gray, A.J. 1985 Pyrethroid structure-toxicity relationships in mammals. Neurotoxicology 3, 25-35

Gray, A. J., Connors, T.A., Hoellinger, H. and Nguyen-Hoang-Nam. 1980. The relationship between the Pharmacolcinetics of intravenous cismethrin and bioresmethrin and their mammalian toxicity. Pestic. Biochem. Physiol. 13, 281-293.

Haigh, J.C. and Haigh, J.M. 1980, immobilizing drug emergencies in humans Vet Hum Toxical 22, 1-5.

Halbach, S. And Clarkson, T.W.(1978). Enzymic oxidation of mercury vapour by erythrocytes. Biochim Biophys Acta 523, 522-531.

Haley, T.J. 1978. A review of the literal of rotenone 1, 2,12,12a tetrahydro-8-9- dimethoxy-(2-(-1 methyl ethenyl)-1-benzo-pyrano (3,5-B)fluoro (2,3-H) (1)-benzo-pyran-6(6h), one J. Enviror pathol. Toxicol 1, 315-337.

Hayes, W.J., Jr (1982). "Pesticides Studies in Man". Williams & Wilkins, Baltimore, Maryland.

Hearn, C.E.D. (1973). A review of Agricultural Pesticide Incidents in Man in England and Wales, 1952-71, Br. J. Ind. Mect 30, 253 -258

Heikkila, E, Nicklas, W.J,vyas,I, and Duvoisin, R.C. 1985. Dopeminergic toxicity of rotenone and the 1 methyl-4-phenylpridium ion after stereotoxic administration to rats:implication for the mechanism of 1-methyl-4-phenyl-1,3,3,6-tetrahydropyridine toxicity. Neurosci. LeH. 62, 389-394.

Herve, J.J. (1985). Agricultural, public health health and animal health usage. In "The pyrethroid insecticides" (J.P. Leahey, ed) Taylor & Francis, London and Philadelphia.

Hoffmann, D; Laboie, E.J. and Hecht, S. 5,1985 Nicotine A precursor for carcinogens. Cancer letter, 26,67-75.

Horiuchi, K, Horiguchi S and suekane, M (1959). Studies on the industrial lead poisoning .I. Absorption, transportation, deposition and excretion of lead 6. The lead contents in organ tissues of the normal Japanese Osaka city med J. 5, 41-70.

Hursh, J.B.(1985). Partition coefficients of mercury (^{203}Hg) vapour between air and biological fluids.J.Appl Toxicol 5,327-332.

Jacques, Y., romey, G., Cavey, M.T., Kartalovski, B. And Lazdunski, M. 1980. Interaction of Pyrethroids with the Na^+ channel in mammalian neuronal cells in culture. Biochim. Biophys. Acta 600, 882-897.

Lawrence, L.J. and Caside, J.E. 1982 Pyrethroid toxicology: Mouse intracerebral structure-toxicity relationships. Pestic Biochem Physiol 18, 9-14.

Lazdunski, M., Barhanin, J., Borsotto, M., Frelin, C., Hugues, M.n Lombet, A., Pauron, D., Renaud, J., Schmid, A. and Vigne, P. 1985 Markers of membrane ionic channels In "Vascular Neuroeffector Mechanisms (J.A Bevan et al. Eds) Elsevier, Amsterdam.

Lehman, A.J, 1954. A toxiocological Evaluation of household insecticides Q.Bull-Assoc. Food Drug Off. 18, 3-13

Lund, A.(1956a). Distribution of thallium in the organism and its dimination. Acta pharmacol. Toxicol 12,251-259.

Lund, A. (1956b). The effect of various substances on the excretion and the toxicity of thallium in the rat. Acta Pharmacol. Toxicol 12, 251-259

Martin, B.R. (1986). Nicotine receptors in the central nervous system. In "The receptors (P.M. comm., ed), vol.3 pp. 393-415. Pergamon Oxford.

Martin, H. And Worthing, C.R; eds (1977). The Pesticide Manual," 5th ed. Br. Crop Rot. Counc; Molvern, Worcestershire, England.

McLaughlin, G.A. 1973. History of Pyrethrum. In "Pyrethrum: The Natural Insecticide" (J.E. Casida, ed), pp 3-15. Academic Press, New York and London.

Metcalf, R.L. (1955). "organic Insecticides" Wiley (intersciences), New York.

Miller, L.K., Lingg, A.J. and Bulla, L.A. 1983. Bacterial, viral and fungal insecticides science 219, 715-721.

Molaughlin, G.A. (1973). Histgory of Pyrethrum. In "Pyrethrum: The National Insecticide" (J.E Casida, ed), pp 3-15. Academic Press, New York and London.

Narahashi, R, 1986. Mechanisms of action of Pyrethroids on sodium and calcium channel gating. In "Neuropharmacology of Pesticide Action" (M.G. Ford, G.G. Lunt, R.C

Reay, and P.N.B. Usherwood, eds), pp 36-40. Ellis Horword, Chichester, U.K.

Nebeker, A.V., Dunn, K.D, Griffis, W.H., Schuytema, G.S. 1994. Effects of dieldrin in food and growth and bioaccumulation in Mallard ducklings Arch Environ Contam. Toxicol 26; 29-32.

Parker, C.M., Albert, J.R., Van Gelder, G.A; Patterson, D.R. and Taylor, J.L. 1985. Neuropharmacologic and neuropathologic effect of Fenvalerate in mice and rats. Fundam.

Rickard, J. and Brodie, M.E. 1985 Correlation of blood and brain levels of the neurotoxic Pyrethroid deltamethrin with the onset of symptoms in rats. Pestic, Biochem. Physiol 23, 143-156.

Schieveibein, H. 1982, Nicotine, resorption and fate pharmacol. Ther 18, 233-248.

Shepard, H.H. 1951. "The Chemistry and action of Insecticides". 1st ed. McGraw-Hil, New York.

Shono, T., Ohsawa, K. And Casida, J.E. 1979. Metabolism of trans- and cis-cypermetrin and decamethrin by microsomal enzymes J. Agric Food Chem. 27, 316-325.

Staatz-Benson, C.G., and Hosko, M.J. 1986. Intataction of Pyrethroids with mammalian spinal neurons. Pestic. Biochem physiol 75, 19-30

Steffeee, C.H and Baetjer, A.M. (1965). Histopathologic effects of chromate chemical Arch. Environ.Health 11,66-75.

Underwood, E.J. (1977)."Trace elements in Human and Animal Nutrition". 4th ed. Academic press, New York.

Verschoyle, R.D. and Aldridge, W.N. 1980 Structure-activity relationship of some Pyrethroids in rats. Pestic. Biochem. Physiol. 2, 308-311.

Van den Bercken, J., Akkermann, L.M.A and van der Zalm, J.J, 1973. DDT. Like action of Allethrin in the sensory nervous system of Xenopus laevis. Eur. J. Pharmacol. 21, 95-106.

Van Emden HF, Pealall DB (1996) Beyond Silent Spring, Chapman & Hall, London, pp 322.

White, I.N.H., Verschoyle, R.D., Moradian, M.H., and Barnes, J.M. 1976. The relationship between brain levels of cismethrin and bioresmethrin in female rats and neurotoxic effects. Pestic. Biochem. Physiol. 6, 491-500.

WHO 1962 Accidental Food Poisoning with Agrosan. Communication to World Health, Organisation from S.A Raz Ali. WHO Inf. Circ. Toxic. Pestic. Man No 9, P. 23

WHO 1972, "IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man," Vol. 1, Int. Agency Res. Cancer, Lyon, France.

Wong, L.C; Heimbach, M.D; Truscolt, D.R. and Duncan, B.D. (1964). Boric acid poisoning. Report of 11 cases. Can.med. Assoc J. 90, 1023.

World Health Organisation (WHO) (1970). "Fluuorides and Human Health". World Health organ. Geneva

World Health Organization (WHO) (19670) "safe use of pesticides in public Health", Who Tech. Rep ser. No 356 world Health Organ, Geneva.

Wright, C.D.P., Forshaw, P.J. and Ray, D.E. 1988 Classification of the actions of two Pyrethroid insecticides in the rat, using the trigeminal reflex and skeletal muscle as test systems. Pestic. Biochem, Physiol. 30, 79-86.

2

Chlorfluazuron as Reproductive Inhibitor

Farzana Perveen
Chairperson, Department of Zoology
Hazara University, Garden Campus
Mansehra
Pakistan

1. Introduction

Benzoyl phenyl ureas (BPUs) inhibit chitin synthesis during growth and development in insects and act as moult disruptors, therefore, they have been called insect growth regulators (Wright and Retnakaran, 1987; Binnington and Retnakaran, 1991). IGRs, such as dimilin, are effective against a considerable range of insect larvae and adults in a variety of situations. The compound disrupts the moulting process by interfering with chitin synthesis. Research on the different aspects of dimilin as a chitin synthesis inhibitor, toxicant, ovicide, disrupting adult emergence, and residual effects have been done with various insect species, e.g., Jakob, 1973; James, 1974; Qureshi et al.,1983; Naqvi and Rub, 1985; Ganiev, 1986; Khan and Naqvi, 1988; Gupta et al., 1991; Tahir et al., 1992; Nizam, 1993. Several modes of action have been reported for these pesticides. For example, phagodeterrents and repellents (Abro et al., 1997), chitin synthesis inhibition (Hajjar and Casida, 1979), growth inhibition and abnormal development (Hashizume, 1988), ovicidal action (Hatakoshi, 1992), insecticidal effects on the reproductive system (Chang and Borkovec, 1980) and neurotoxic effects on insect behaviour (Haynes, 1988).

1.1 Chlorfluazuron

Chlorfluazuron (Atabron®) is a benzyl phenyl urea (BPU) chitin-synthesis inhibitor (CSI) and insect growth regulator (IGR) is formed by Ishihara Sangyo Kaisha, Japan. Some important details concerning the insecticide chlorfluazuron are given below (provided by Ishihara Sangyo Kaisha, Japan) (Perveen, 2005):

Common name	:	Chlorfluazuron (proposed to ISO)
Other names	:	Atabron® or Helix® or Aim®
Source	:	Ishihara Sangyo Kaisha Ltd., Tokyo, Japan
Code number	:	IKI-7899, CG-112913, pp-145
Formulation type	:	5% w/w (Emulsifiable concentration: EC)
Chemical name	:	[1-{3,5-dichlor-4-(3-chlor-5-trifluoromethyl-2-pyridyloxy) phenyl}-3-(2,6-ifluorobenzoyl) urea] (IUPAC nomenclature)

1.1.1 Salient physical and chemical properties (Perveen, 2005)

Appearance	:	Crystalline solid at 20 °C
Odor	:	Odorless

Melting point	:	222.0 – 223.9 °C (decomposes after melting)
Vapor pressure	:	<10 – 8 p_a, <10-10 Torr at 20 °C
Volatility	:	Relatively non-volatile
Specific gravity	:	1.4977 at 20 °C
Stability	:	No detectable decomposition over at least 3 months at 50 °C

1.2 Spodoptera litura

The *S. litura* is found in most of the Caroline and in the South Pacific Island including American Samoa. It also occurs in the northern two thirds of Australia. The moth is also widespread throughout India and recognized as quarantine pest in EU legislation. It is present in Mediterranean Europe and Africa. It is the most commonly intercepted in the UK, on imported ornamentals and their products. *Spodoptera litura* is also a destructive pest of subtropical and tropical agriculture, and has the potential to be a serious pest of glasshouse crops in northern Europe. It was found as feeding on impatience on Victoria Peak on Hong Kong Island and readily switched to (western) lettuce (Etman and Hooper, 1979). In 1974, Etman and Hooper initiated an investigation into the radiobiology of *S. litura*, and reported that it was a significant pest of cotton in the Ord River region, Australia (Etman and Hooper, 1979). Its larvae are a major cosmopolitan pest of a wide range of crops (Skibbe et al., 1995). Matsuura and Naito (1997) reported that *S. litura* causes serious widespread damage to many agricultural crops in the far southern of the Central Japan every year. They hypothesized that adult *S. litura* immigrate into Japan from overseas every year by long-distance migration. The larvae are destroyed many economically important crops such as *Gossypium hirsutum* L., *Brassica oleracea* L., *Spinacea oleracea* L., *Trifolium alexandrinum* L., *Medicago sativa* L., *Arachis hypogaea* L., *Phaseolus aureus* Roxb., *Phaseolus vulgaria* L. and *Nicotiana tabacum* L. during different seasons throughout the year in Pakistan (Younis, 1973). Their larvae eat nearly all types of herbaceous plants. Some examples of plants are: tobacco, *Nicotiana tabacum* L.; tomatoes, *Lycopersicum esculentum* Mill.; cauliflower, *Brassica botrytis* L.; beetroot, *Beta vulgaris conditiva* L.; silver beet (swiss chard) *Beta vulgaris cicla* (L.); peanuts, *Arachis hypogaea* L.; beans, *Phaseolus vulgaris* L.; banana, *Musa* paradisiaca L.; strawberry, *Fragaria* vesca L.; apple, *Malus pumila* Mill.; lettuce, *Lactuca sativa* L.; zinnia, *Zinnia elegans* Jacq.; dahlia, *Dahlia pinnata* Cav.; ape, *Alocasia macrorrhiza* (L.); geranium, *Pelargoniumx zonale*; St. John's lily, *Crinum asiaticum* L.; mangrove lily, *Crinum pedunculatum* (Fragrant); leek, *Allium porrum* (Leek); horsetail she oak, *Casuarina equisetifolia* L.; *Fuchsia* and many other garden plants (Baloch and Abbasi, 1977). Several common names have been used for *S. litura*, for example defoliator cutworm, oriental leafworm, cluster caterpillar and common cutworm. The larvae are quite polyphagous for example eat all types of herbaceous plants and have been reaching the status of international pest. In 1968, a panel convened by the International Atomic Agency listed species of *S. litura* as a pest on which basic and applied research was needed in order to evaluate the potential of the sterile insect release method for control (Anonymous, 1969).

Eggs of *S. litura* are laid in batches, on plants and other surfaces such as pots, benches or glasshouse structures. Eggs are normally laid in the irregular furry masses covered with orange-brown hairs giving them a "felt-like" appearance on the underside of a leaf of a food plant similar to the egg of the brown locust, *Locustana pardalina* (Walk.) (Matthee, 1951). On hatching, larvae (caterpillars) are 2–3 mm long with white bodies and black heads and are very difficult to detect visually. If they emerge from eggs laid on glasshouse structures or

hanging pots, they can reach the plants below by "parachuting" down on silken threads. The overall colour of the later-stages of the larvae can vary from light to dark brown, and the body is strongly speckled with tiny white spots. Initially, when larvae grow become a translucent green with a dark thorax. The young larvae are smooth-skinned with a pattern of red, yellow, and green lines, and with a dark patch on the mesothorax. Larvae initially eat only the flesh of their food leaves, leaving the veins intact. Later, as they grow, they eat whole leaves, and even flowers and fruit (Khuhro et al., 1986).

Many populations are extremely resistant to pesticides and, if they become well established, can be exceptionally difficult to control. In these cases, it is important that a comprehensive treatment programme is implemented, incorporating a range of reliable control methods, including physical destruction of insects. The *S. litura,* as it is the most common to be encountered in a UK nursery, but the larvae and adults of all noctuids are similar in appearance and are difficult to tell apart without laboratory examination (Khuhro et al., 1986). Larvae become brown with three thin yellow lines down the back, one in the middle and one on each side. A row of black dots runs along each side, and a conspicuous row of dark triangles decorates each side of the back. The last-instar larva is very dark, with four prominent yellow triangles on the mesothorax. When disturbed, the larvae curl into a tight spiral with the head protected in the centre. Larvae further develop characteristic markings on their backs. These include: a square of four yellow spots, each on a black patch, located just behind the head; a further pair of black patches just behind these, and another pair of black patches towards the end of the larva; typically, there are three orange-brown lines, punctuated with dashes of black and yellow along the back of the body. Depending on the background colour, these markings may be more evident on some larvae than others; the larvae ultimately grow up to 4.5 cm long; larvae are nocturnal, and during the day can be found at the base of plants or under pots. The feeding activity of young larvae causes "windows" in the leaves, while older larvae can completely defoliate plants if present in large numbers. Stems, buds, flowers and fruits may also be damaged. The larvae burrow into the soil below the plant for several centimeters and pupate there without a cocoon. As they do so, they produce a quantity of fluid, and drown in this if they pupate in captivity in an empty glass jar. They pupate successfully if 0.5 cm of sand is provided in the container. In January in Melbourne, the pupal stage lasts three weeks, but larvae that pupate at the end of summer emerge the following spring. The red-brown pupae are up to 2 cm long. The thoracical, ventral knobs found on covering of pupa (Khuhro et al., 1986). Adult moths with brown colour are up to 2 cm long with a wingspan of approximately 4 cm. The fore-wings are brown, with a large number of pale cream streaks and dashes and, when the adults are newly emerged, there may be a violet tint to the fore-wing. The hind-wings are a translucent white, edged with brown. The hind-wings are silvery white. It has a wingspan of about 4.0 cm. The males but not the females have a blue-grey band from the apex to the inner margin of each forewing. The pheromones of this species (specific sex-attractant scents used by females to attract males) have been elucidated. As the adults are nocturnal, light or pheromone traps should be used for monitoring purposes. Seek assurance from suppliers that plants are free from this pest as part of any commercial contract: carefully inspect new plants and produce on arrival, including any packaging material, to check for eggs and larvae and for signs of damage (Khuhro et al., 1986).

Early notification of the presence of this pest, will allow rapid implementation of a comprehensive treatment programme, and will help eradicate it quickly from nursery. Established outbreaks are very damaging and difficult to eradicate. Various methods of

control of *S. litura* have been investigated. Biologically, it has been controlled by the nematode, *Steinernema carpocapsae* (Weiser) and parasitoid fly, *Exorista japonica* (Townsend). A baculovirus has also been used. Resistant species of plants are also grown to save the crops from this pest. Resistant tomatoes are most commonly cultivated (Khuhro et al., 1986).

2. Effects of chlorfluazurn on reproduction of *Spodoptera litura*

Reproductive inhibition induced by BPUs has been reported the most widely when applied to adults or eggs of insect pests rather than to application to larvae or pupae (Fytizas, 1976). When these compounds were applied to females, males or both sexes of insect pests, BPUs induced a variety of effects on reproduction; they caused a decrease in fecundity, fertility and/or hatchability. It has been reported that treatment of adult insect pests with diflubenzuron disrupts the secretion of adult cuticle (Hunter and Vincent 1974; Ker, 1977), and the production of peritrophic membrane in the grasshopper, *Locusta migratoria* (L.) (Clark et al., 1977) and the meal worm, *Tenebrio molitor* L. (Soltani, 1984; Soltani et al., 1987). In addition, topical treatments of male and female adult boll weevils, *Anthonomous grandis* Boheman; stable flies, *Stomoxys calcitrans* (L.) and *M. domestica* with TH-6040 [{N-(4-chlorophenyl)-N-26-difluorobenzoyl}urea] caused significant reduction of egg fertility and hatchability. It also causes inhibition in the fecundity of female adults of several species of insect pests (Holst, 1974; Taft and Hopkins, 1975; Crystal, 1978; Hajjar and Casida, 1979; Otten and Todd, 1979). Diflubenzuron applied to adult females caused a decrease in fecundity in the Mexican bean beetle, *Epilachna varivestis* Mulsant and Colorado potato beetle, *Leptinotarsa decemlineata* Say (Holst, 1974), and adversely affected egg viability in *St. calcitrans* and *M. domestica* (Wright and Spates, 1976). When 2 day-old female adults of the Japanese beetle, *Oryzae japonica* Willemse, were starved for 6 hours, and then allowed to consume 500 µg a.i. of diflubenzuron over another 6 hours, the fecundity of the treated females, in term of number of eggs laid per pod, was significantly decreased from controls. In controls, most pods gave an egg hatch of 82.5% but a hatch of only 8.5% hatched in the treated females (Lim and Lee, 1982). Similarly, treating eggs with diflubenzuron caused reduction in hatching in the mosquitoes, *Culex pipiens* Say, *C. quinquefascialus* Say (Miura et al., 1976), the almond moth, *Ephestia cautella* (Walk.) (Nickle, 1979), the two-spotted lady beetle, *Adalia bipunctata* (L.) and the seven-spotted lady beetle, *Coccinella septempunctata* L. (Olszak, 1994). Reports of inhibition of reproduction when larvae or pupae (instead of adults or eggs) were treated with BPUs are rare and little literature is available (Madore et al., 1983). Brushwein and Granett (1977), working with the spruce budworm, *Choristoneura fumiferana* (Celemens), demonstrated that certain moult-inhibiting IGRs such as EL-494 (Eli Lilly and Co., New York, USA) fed to sixth-instar larvae, caused reproductive failure in adults surviving after the larval treatment. Therefore, in this research newly ecdysed fifth-instar larvae and newly ecdysed pupae of *S. litura* were used as test materials. Chlorfluazuron, a comparatively new IGR and BPU that was discovered by Ishihara Sangyo Kaisha Ltd., Tokyo, Japan, that has been developed and sold commercially as Atabron®, Helix® and Aim® in many countries, including Japan in cooperation with Novelties Co. Ltd, ICI-AGRO and Ciba Geigy. It is a relatively highly active chitin synthesis inhibitor and it is, therefore, an effective treatment for the control of major lepidopteran insect pests in crops such as cotton, fruits, tea, vegetables and where insect resistance to conventional insecticides is becoming a serious problem. Chlorfluazuron exhibits no activity against important beneficial insects (Haga et al., 1992). The highly selective insecticidal activity of

chlorfluazuron is particularly suited to integrated pest management programmes. Although chlorfluazuron has contact toxicity at higher rates, the major route of toxicity to insects is ingestion, and it has no root, systemic or foliar translaminar activity. Like other BPUs, chlorfluazuron is believed to disrupt chitin formation and, thus, kills the insects when they moult. This mode of action necessarily means that it is effective only against immature insects and that it is relatively slow actions. When higher dosages of chlorfluazuron were applied to newly ecdysed fifth-instar larvae, it had a devastating effect on the *S. litura* population by killing them during larval, pupal and adult stages (Hashizume, 1988). In insect pest management, the purpose of research is to maintain the pest population below the economic injury level. The mode of action of chlorfluazuron, as a CSI is known to some extent. However, but the knowledge of its effects on reproduction are rare. Insect structure and physiology may vary considerably during growth and development, with certain stages being more susceptible to insecticides than others. For example, the cuticle varies in its composition during larval development and this has been related to changes in IGR susceptibility. The activity of various insecticides detoxifying enzymes, such as MFO, glutathione S-transferase and epoxide hydrase also fluctuate during the life cycle of an insect (Yu, 1983). For this purpose newly ecdysed fifth-instar larvae and newly ecdysed pupae were selected for the treatments for the present research. The main objective of the present research is to determine the effects of sublethal doses (LD_{10}: 1.00 ng larva^{-1}; 0.12 ng female pupa^{-1}; 1.23 ng male pupa^{-1} or LD_{30}: 3.75 ng larva^{-1}) of chlorfluazuron on the reproduction (e.g., fecundity, fertility and hatchability) when ha been apply to newly ecdysed fifth-instar larvae and newly ecdysed pupae of *S. litura* (Perveen, 2000a).

2.1 Experimental procedures

2.1.1 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 diameter: 4×4 cm high) for hatching under the same environmental conditions (Perveen, 2000a).

2.1.2 Chlorfluazuron and its application

Sublethal doses, LD_{10} (1.00 ng larva^{-1}; 0.12 ng female pupa^{-1}; 1.23 ng male pupa^{-1}) or LD_{30} (3.75 ng larva^{-1}) were applied to newly ecdysed fifth-instar larvae and newly ecdysed pupae. These LD_{10} and LD_{30} values were calculated based on interpret alone of the results of the toxicity data of larval tests at adult emergence. The treated and untreated insects, at all developmental stages including fifth- and sixth-instar larvae, pupae and adults, were weighed separately, on different developmental days, using an analytical balance (Sartorius Analytical AC-2105, Tokyo, Japan) to a precision of 0.001 mg, to determine the effect of chlorfluazuron on the body weight. The duration of each developemental stage was also strictly recorded (Perveen, 2000a).

2.1.3 Mating

After both larval and pupal treatments, females and males that emerged between 2 and 8 hour (most adults emerged in the dark photoperiod) on the same day were collected at 0200–1000 hour. These adults were considered as 0 day old and paired just before the dark photoperiod (12 hour old) of the next day. Each female and male pair was kept separately in a plastic cup (430 cm^3; height: 8.0 cm; diameter: 9.5 cm) for the whole life-span. The cup was padded with Rido cooking paper on its wall and with a disc of 70 mm filter paper on the bottom. The pairs were fed throughout their life by cotton wool soaked in 10% sugar solution in small plastic cups. All *S. litura* were examined daily (Perveen, 2000a).

To determine the effects of sublethal doses of chlorfluazuron on reproductivity, seven different mating combinations of female and male crosses were established. These were: (1) Untreated female mated with untreated male ($_{U}♀\times_{U}♂$); (2) LD_{10}-treated female mated with untreated male ($_{LD10}♀\times_{U}♂$); (3) Untreated female mated with LD_{10}-treated male ($_{U}♀\times_{LD10}♂$); (4) LD_{10}-treated female mated with LD_{10}-treated male ($_{LD10}♀\times_{LD10}♂$); (5) LD_{30}-treated female mated with untreated male ($_{LD30}♀\times_{U}♂$); (6) Untreated female mated with LD_{30}-treated male ($_{U}♀\times_{LD30}♂$); (7) LD_{30}-treated female mated with LD_{30}-treated male ($_{LD30}♀\times_{LD30}♂$). For the fecundity, fertility and hatchability experiments, 15–30 pairs were used for each cross. Eggs were laid on the cooking paper after 24 hours and were collected during 0800–1000 hour, cut out and kept in cups (90 cm^3) for hatching. Eggs laid were hatched within 84 hours. Observation of oviposition continued until the death of female. Four days after each collection of eggs, the fecundity, fertility and hatchability of the laid eggs were assessed. After the natural death of females, the spermatophores were separated from the bursa copulatrix with a fine forceps in 0.9% NaCl (Saline or Ringer's solution: Barbosa, 1974) under the binocular microscope (10×magnification) (Olympus Co. Ltd., Tokyo, Japan) (Perveen, 2000a).

2.1.4 Data analysis

Data for the effects of sublethal doses of chlorfluazuron on reproductivity and viability were analyzed using analysis of variance, one way ANOVA (Concepts, 1989; Minitab, 1997; Walpol and Myers1998) at $P<0.0001$ and Scheffe's F-test (multiple range) (Scheffe, 1953) at 5%. Hatchability percentage values were normalized by arcsin transformation before statistical analysis (Anderson and McLean, 1974).

2.2 Results

When the LD_{10} (1.00 ng $larva^{-1}$; 0.12 ng female $pupa^{-1}$; 1.23 ng male $pupa^{-1}$) and the LD_{30} (3.75 ng $larva^{-1}$) of chlorfluazuron were applied to newly ecdysed fifth-instar larvae or newly ecdysed pupae, it was observed that the fecundity of the resulting adults and the fertility and hatchability of their eggs, was significantly reduced {$P<0.0001$ (for larval treatment); $P<0.05$ (for pupal treatment)}, compared with untreated adults, but no significant differences were observed between larval and pupal treatments ($P<0.02$) (Tables 1 and 2). When chlorfluazuron was applied to newly ecdysed fifth-instar larvae at sublethal doses, the number of eggs oviposited by a treated females mated with an untreated male ($_{T}♀\times_{U}♂$) was suppressed to the same degree as an untreated female mated with a treated male ($_{U}♀\times_{T}♂$) or a treated female mated with a treated male ($_{T}♀\times_{T}♂$) (Table 1). The mean female fecundity was 2250±198 eggs when both male and female were untreated (control), i.e., ($_{U}♀\times_{U}♂$), (Table 2). When the female was treated either by the LD_{10} or LD_{30} and mated

with an untreated male, the fecundity was 1462±353 ($_{LD10}♀\times_{U}♂$) and 1266±237 ($_{LD30}♀\times_{U}♂$), respectively. When the male was treated with the LD_{10} and mated with an untreated female, the fecundity was 1407±334 ($_{U}♀\times_{LD10}♂$). However, in the same cross when the male was treated with LD_{30} instead of the LD_{10} ($_{U}♀\times_{LD30}♂$), the fecundity was 1270±215. When both sexes were treated with the LD_{10} ($_{LD10}♀\times_{LD10}♂$), it was 1330±295 and when both sexes were treated with the LD_{30} concentration ($_{LD30}♀\times_{LD30}♂$), it was 1331 ±295. In all the crosses, the fecundity was significantly reduced when compared with the control cross ($_{U}♀\times_{U}♂$) (Table 1) (Perveen, 2000a).

Mating pairs[a] (female×male)	n[a]	Fecundity[b,c] (mean±SD)	Fertility[b,c] (mean±SD)	Hatchability %[c,d] (mean±SD)
$_{U}♀\times_{U}♂$	30	2250±198a	1984±208a	88.4±6.6a
$_{LD10}♀\times_{U}♂$	30	1462±353b	1010±315b	68.9±11.8b
$_{U}♀\times_{LD10}♂$	28	1407±334b	688±317c	48.3±17.1c
$_{LD10}♀\times_{LD10}♂$	30	1330±295b	643±265c	48.6±14.2c
$_{LD30}♀\times_{U}♂$	29	1266±237b	828±206b	65.8±11.4b
$_{U}♀\times_{LD30}♂$	30	1270±215b	36±155d	28.8±11.2d
$_{LD30}♀\times_{LD30}♂$	29	1331±295b	33±121d	27.1±9.1d

[a]LD_{10}: 1.00 ng larva^{-1}; LD_{30}: 3.75ng larva^{-1}; U: untreated (control); ♀: female adults; ♂: male adults; n: number of pairs used

[b]Number of eggs oviposited during the whole life of female adult were counted (fecundity) and from the eggs number of hatched that larvae were counted (fertility).

[c]Data were analyzed using one way ANOVA (Concepts, 1989) at $P<0.0001$). Means within columns followed by different letters indicate significant differences by Scheffe`s F-test (Scheffe, 1953) at 5%.

[d]Hatchability % values were normalized by arcsin transformation before statistical analysis (Anderson and McLean 1974).

Table 1. Effects of sublethal doses of chlorfluazuron on fecundity, fertility and hatchability after topical application of newly ecdysed-fifth instar larvae of *Spodoptera litura* (Source: Perveen, 2000a).

When the LD_{10} of chlorfluazuron was applied to newly ecdysed pupae and the resulting adults were paired, the control fecundity was 2170±175. It was not significantly reduced ($P<0.02$) compared with the larval treatment. The fecundity in the treated pupal cross ($_{LD10}♀\times_{U}♂$) was 640±83. It was not significantly reduced ($P<0.02$) compared with the same cross with treated larvae. In the pupal treatment, the fecundity was suppressed to a similar degree in the crosses $_{LD10}♀\times_{U}♂$, $_{U}♀\times_{LD10}♂$, $_{LD10}♀\times_{LD10}♂$. This reduction was significant ($P<0.0001$) when compared with the control cross (Table 1) (Perveen, 2000a). There was no significant reduction ($P<0.02$) between the larval and pupal treatments with respect to fecundity (Tables 1 and 2) (Perveen, 2005).

The mean fertility of females was 1984±208 larvae when both the male and female were untreated, i.e. the control ($_{U}♀\times_{U}♂$) (Table 1). When the female was treated with either the LD_{10} or LD_{30} and mated with an untreated male, the fertility was ($_{LD10}♀\times_{U}♂$: 1010±315; $_{LD30}♀\times_{U}♂$: 828±206, respectively), i.e., significantly reduced compared with the control cross ($_{U}♀\times_{U}♂$). When the male was treated with the LD_{10} and mated with an untreated female, the fertility ($_{U}♀\times_{LD10}♂$: 688±317) was significantly reduced compared with the crosses, i.e.,

$_{LD10}$♀×$_{U}$♂ and $_{LD30}$♀×$_{U}$♂. However, in the same cross when the male was treated with the LD_{30} instead of LD_{10}, the fertility was ($_{U}$♀× $_{LD30}$♂: 368±155) significantly lower than the $_{U}$♀×$_{LD10}$♂ cross. When both sexes were treated with the LD_{10}, the fertility was ($_{LD10}$♀×$_{LD10}$♂: 643±265) not significantly different from the $_{U}$♀ ×$_{LD10}$♂ cross. Similarly, when both sexes were treated with the LD_{30}, the fertility was ($_{LD30}$♀×$_{LD30}$♂: 333±121) not significantly different from the $_{U}$♀×$_{LD30}$♂ cross (Table 1) (Perveen, 2000a).

Mating pairs[a] (female×male)	n[a]	Fecundity[b,c] (mean±SD)	Fertility[b,c] (mean±SD)	Hatchability %[c,d]
$_{U}$♀×$_{U}$♂	15	2170±175a	2123±177a	97.8a
$_{LD10}$♀×$_{U}$♂	15	1640±83b	1090±79b	66.5b
$_{U}$♀×$_{LD10}$♂	15	1580±75b	827±49c	52.3c
$_{LD10}$♀×$_{LD10}$♂	15	1524±76b	751±51c	49.3c

[a]LD_{10}: 0.12 ng female pupa^{-1}; 1.23 ng male pupa^{-1}; n: number of pairs used
[b]Number of eggs oviposited (fecundity) the during whole life of female adults were counted and from the number of eggs that hatched larvae were counted (fertility).
[c]Data were analyzed using one-way ANOVA (Concepts, 1989) at $P < 0.001$. Means within a column followed by different letters indicate significant differences according to Scheffe's F-test (Scheffe, 1953) at 5%.
[d]Hatchability% values were normalized by arcsin transformation before statistical analysis (Anderson and McLean 1974).

Table 2. Effects of a sublethal doses of chlorfluazuron on fecundity, fertility and hatchability after topical application of newly ecdysed pupae of *Spodoptera litura* (Source: Perveen, 2005).

When the LD_{10} of chlorfluazuron was applied to newly ecdysed pupae and resulting adults were paired, the fertility of the control cross, $_{U}$♀×$_{U}$♂ was 2123±177, which was not significantly reduced (P<0.02) than that in the larval treatment. In the same way, with the $_{LD10}$♀×$_{U}$♂ cross, the fertility was 1090±79, which was not significantly reduced (P<0.02) than the same cross in the larval treatment. The fertility was reduced 61 –65% in the $_{U}$♀×$_{LD10}$♂ cross, which was significantly reduced (P<0.02) than the $_{LD10}$♀×$_{U}$♂ cross with a fertility reduction of 46–49%. The fertility of the $_{U}$♀×$_{LD10}$♂ cross was not significantly reduced (P<0.02) than the $_{LD10}$♀×$_{LD10}$♂ cross, which was reduced 65–68% for both larval and pupal treatments. There were no significant reductions (P<0.02) between larval and pupal treatments with respect to fertility (Tables 1 and 2). The mean hatchability during a female life-span was 88.4(±6.6)% when both male and female were untreated, i.e. the control ($_{U}$♀×$_{U}$♂) cross (Table 1). When the female was treated, either by the LD_{10} or LD_{30} and mated with an untreated male, the hatchabilities were 68.9 (±11.8)% ($_{LD10}$♀×$_{U}$♂) and 65.8(±11.4)% ($_{LD30}$♀×$_{U}$♂), respectively significantly reduced compared with the control cross. When the male was treated with the LD_{10} and mated with an untreated female, the hatchability, 48.3(±17.1)% ($_{U}$♀×$_{LD10}$♂) was significantly reduced than the $_{LD10}$♀×$_{U}$♂ and $_{LD30}$♀×$_{U}$♂ crosses. However, in the same cross when the male was treated with LD_{30}, instead of LD_{10}, hatchability, 28.8(±11.2)% ($_{U}$♀×$_{LD30}$♂), significantly reduced than the $_{U}$♀×$_{LD10}$♂ crosses. When both sexes were treated with LD_{10}, the hatchability was 48.6(±14.2)% ($_{LD10}$♀×$_{LD10}$♂), not significantly different from the $_{U}$♀×$_{LD10}$♂ cross. Similarly, when both sexes were treated with LD_{30}, the hatchability was 27.1(±9.1)% ($_{LD30}$♀×$_{LD30}$♂), which was not significantly different from the $_{U}$♀×$_{LD30}$♂ cross (Table 3.1). The hatchability for the $_{U}$♀×$_{U}$♂ cross was 88.4% and

97.8%, respectively, for the larval and pupal treatments. The larval-treated cross, $_{LD10}♀\times_{U}♂$, was 68.9%, which is not significantly reduced (P<0.02) than the same cross of the pupal treatment in which the hatchability was 66.5%. The hatchability was reduced 48.7% and 52.7% in the $_{U}♀\times_{LD10}♂$ cross, respectively, for the larval and pupal treatments. In the $_{LD10}♀\times_{LD10}♂$ cross, it was reduced to 48.6% and 49.3%, respectively, for these treatments. There was no significant reduction (P<0.02) between larval and pupal treatments with respect to hatchability (Tables 1 and 2) (Perveen, 2000a).

2.3 Discussion

When chlorfluazuron was applied to newly ecdysed fifth instars at sublethal doses, LD_{10} (1.00 ng larva^{-1}) or LD_{30} (3.75 ng larva^{-1}), it was observed that the fecundity of resulting adults as well as the hatching rate of their eggs was suppressed. The hatching rate of eggs oviposited by an untreated female mated with a treated male was suppressed to the same degree as that ofeggs oviposited by a treated female mated with a treated male. However, Madore et al. (1983) studied the effects when different concentrations of sublethal doses of the UC-62644 (chlorfluazuron-25) fed to sixth instar larvae of spruce budworm. Homologous crosses between adults of the 0.01, 0.025 and 0.034 ppm treatments showed 0, 69 and 97% reduction, respectively, in the numbers of eggs laid per 30 pairs of moths when compared with control. Emam et al. (1988) reported the fecundity of *S. littoralis* adults decreased significantly from 977.64 eggs in control to 421.75 eggs, a decrease of about 56%, for adults feeding 10% honey solution containing 0.5 p.p.m. chlorflufluazuron. The corresponding fertility inhibition amounted to 32%. In the present case the fertility was significantly different when only the female was treated or only the male was treated. It is obvious from the results that the fertility and hatchability were affected more when the male was treated in comparison with the female, as also reported by Abro et al. (1997), who found that males were more sensitive to insecticides than females, when five concentrations of cyhalothrin and fluvalinate were tested against fourth instar larvae of *S. litura*.

2.4 Conclusion

To clarify the sublethal effects of chlorfiuazuron on reproductivity of common cutworm, *Spodoptera litura*, experiments were conducted under laboratory conditions. Reduction in the body weight was 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. When sublethal doses were applied only to females or only to males, or both sexes, the average fecundity reduction was up to 35–44%. When only females were treated with sublethal doses, fertility was reduced by 49–58%; when only males were treated fertility was reduced by 65–81% and when both sexes were treated, fertility was reduced by 68±83%. Hatchability was reduced by 22–26% when only females were treated, by 44–66% when only males were treated and by 45–72% when both sexes were treated with LD_{10} or LD_{30} doses as newly ecdysed fifth instars. 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}. Currently, work is in progress to find out the main reasons for the sublethal effects of chlorfluazuron on reproductivity and viability.

3. Effects of chlorfluazurn on female reproductive system of *Spodoptera litura*

In many insects oviposition requires the development of the ovary, egg maturation, mating and, in some insects, feeding of the females. Ovarian development, which includes oöcyte growth and vitellogenesis, is under the hormonal control, of either juvenile hormone or ecdysteroid (Engelmann, 1979). In many insects, juvenile hormone (JH) regulates the biosynthesis and uptake of vitellogenin by the oöcytes. Among Lepidoptera, e.g., the tobacco hawkmoth, *Manduca sexta* L. (Sroka and Gilbert, 1971; Nijhout and Riddiford, 1974) and the large white butterfly *Pieris brassica* L. (Karlinsky, 1963 and 1967; Benz, 1969), juvenile hormone is required for full development of the ovaries in adults, whereas in the silkworm *Bombyx mori* L. (Chatani and Ohnishi, 1976), giant silk moth, *Hyalophora cecropia* (L.) (Williams, 1952; Pan, 1977), ailanthus silkmoth, *Samia cynthia* (Drury) (Takahashi and Mizohata, 1975) and ricemoth, *Corcyra cephalonica* (Stainton) (Deb and Chakarvorty, 1981) ovarian development occurs as part of adult development initiated by ecdysteroid. Juvenile hormone or juvenile hormone analogue (JHA) application at a critical period, however, induces abnormal development of the ovary as well as other tissues, although juvenile hormone analogues can replace natural juvenile hormone in regulating oöcyte maturation (Nomura, 1994). In the normal state, the ovary develops during one day before and after eclosion in the presence of juvenile hormone (as described above) and a haemolymph factor stimulates the ovary to start oviposition. When S-71639 was applied to pupae, it inhibited adult emergence when a relatively a high dose was applied. If adults did emerge, they could not oviposit through inhibition of the haemolymph factor, hatchability was also reduced (Hatakoshi and Hirano, 1990). The effects of diflubenzuron on fecundity resulted from treatment of adult females by contact or ingestion (Leuschner, 1974; Fytizas, 1976). When *O. japonica*, adults females were fed diflubenzuron, it retarded the maturation of oöcytes (Lim and Lee, 1982). In *T. molitor*, diflubenzuron reduced mealworm longevity (Soltani et al., 1987), the number of oöcytes per ovary, the duration of the oviposition period and the fecundity (Soltani, 1984). Diflubenzuron, topically applied (0.5 µg insect^{-1}) to codling moth, *Cydia pomonella* L. on pupal ecdysis, inhibited the growth and development of oöcytes. It delayed adult emergence and caused a decrease in both the thickness of the follicular epithelium and the size of the basal oöcytes during pupal development. On the other hand, the size of basal oöcytes, the protein content per ovary and the number of oöcytes per ovary recorded in newly emerged adults were significantly reduced by the diflubenzuron treatment. These results, together with observations in several other species, indicated that the reduction in fecundity and egg viability was probably due to interference by diflubenzuron with vitellogenesis (Soltani and Mazouni, 1992). Under laboratory conditions, effects of topical application of sublethal doses of chlorfluazuron (LD_{10}: 1.00 ng larva^{-1} or LD_{30}: 3.75 ng larva^{-1}) on newly ecdysed fifth-instar on fecundity, fertility and hatchability have been investigated. Thus, it is investigated the causes of the decrease in these parameters. To obtain more information, sublethal doses of chlorfluazuron topically have been applied to newly ecdysed fifth-instar larvae of *S. litura* and the effects on female reproductive system during ovarian development and oögenesis have been observed.

3.1 Experimental procedure

3.1.1 Ovary measurement

Experimental *S. litura* were rared in the same way as mentioned in Section 2.1.1. Sublethal doses, LD_{10} (1.00 ng larva^{-1}; 0.12 ng female pupa^{-1}; 1.23 ng male pupa^{-1}) or LD_{30} (3.75 ng

larva^{-1}) were applied to newly ecdysed fifth-instar larvae same mentioned in Section 2.1.2. To determine the effects of chlorzuazuron on the ovaries, control and treated batches of insects, collected from the fifth day after pupation to the seventh day after adult emergence, were used, depending on the experiment requirements. Ovaries were dissected from these insects in Ringer's solution under a binocular microscope (10×magnifcation: Nikon, Nippon Kogaku, Tokyo, Japan), and the lengths of pedicle, vitellarium, and germarium of each ovariole were measured. The number of mature oöcytes in each of the ovarioles was also counted. The Ringer's solution was removed, and the freshly dissected ovaries were placed in a small covered container that had been preweighed. The dissected ovaries were weighed on an analytical balance (AC-205, Sartorius Analytical, Tokyo, Japan) and kept in the same container in the oven for 24 h at 62±1 °C for evaporation of water. The dried ovaries were reweighed (Perveen and Miyata, 2000).

3.1.2 Histology

The procedure to stain the nuclei for cell density from the germarium during female adults required age was adapted from the method described by He (1994). First, the germarium of the female were removed and kept on a microscope slide and carefully crushed with micro forceps until it was extended and roughly evenly distributed over the slide. Second, several drops of 3:1 methanol-acetic acid were introduced to the slide to fix the preparation for 15 min and then the excess fixing solution was absorbed with a filter paper after the fixation. Third, several drops of a 2–5% Giemsa solution dissolved by Sorensen-Gomori buffer solution (monobasic and dibasic sodium phosphate, 0.07 M, pH 6.8) were introduced to the slide for 10–30 min to stain the preparation. Then after the staining, the slide was washed gently and carefully and dried in the air. Finally, the air-dried preparation was checked with a phase-contrast microscope at 20×magnification (Perveen and Miyata, 2000).

The length and width of the basal oöcytes were measured from the 5th d after pupation to 0 day after adult emergence in control and treated insects. Oöcyte measurements were made on three to four basal oöcytes per pair of ovaries that were taken from 9-10 insects. Measurements were made with a graduated slide under the phase contrast microscope at 400×magnification (BH_2, Olympus, Tokyo, Japan). The size of basal oöcytes was calculated by the formula used by Loeb et al. (1984) for the size of a prolate spheroid, $4/3\pi(ab^2)$, where a is the radius of the long and b is the short dimension of the same oöcyte (Perveen and Miyata, 2000).

The thickness of the follicular epithelium of basal oöcytes was observed by making a parafilm microtomy conducted according to the method described by Yoshida (1994). Basal oöcytes were fixed in Carnoy's solution for 3 hour, washed in 70% ethanol for 2 hour, and dehydrated in an alcohol series 70, 80, 90, and 95% and twice in 100%, followed by a benzene and ethanol solution (1:1), each for 30 min. Incubation was done three times at 60 °C in benzene and paraffin (1:1), and then in parafin only, each for 30 min. Five-mm microtome sections were cut into a rolling ribbon. It was stained in xylene I (10 min), xylene II (5 min), followed by an ethanol series of 100, 90, 80, and 70%, each for 5 min, Mayer's hematoxylin for 15 min, and washed under running water. Microtome sections were mounted in 1% eosin (10 min), distilled water (2 sec), followed by an alcohol series 50, 70, 80, and 90%, and twice in 100% (each for 1-5 min), xylene and 100% ethanol (1:1; 5 min), xylene I (5 min), xylene II (5 min). Finally, the sections were embedded on microscopic graduated slide in a drop of Canada balsam. The microscopic graduated slide was covered with a glass cover slip. The thickness of follicular epithelium was measured under a phase

contrast microscope (BH_2, Olympus, Tokyo, Japan) at 400×magnifcation (Perveen and Miyata, 2000).

3.1.3 Data analysis

Data were analyzed using analysis of variance, one way ANOVA (Concepts, 1989) at $P<0.01$ and Scheffe's *F*-test (Scheffe, 1953) at 5%.

3.2 Results

The morphology of the adult female reproductive system of *S. litura* is shown in Figure 1.

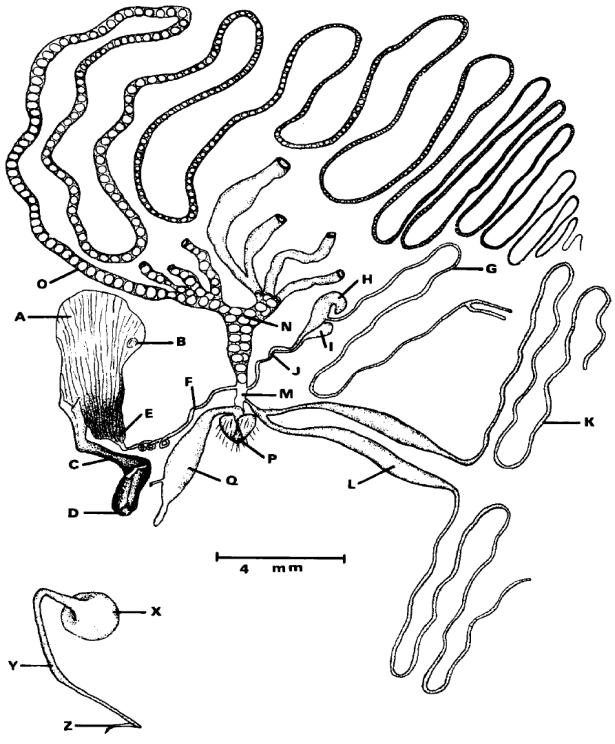

Fig. 1. The morphology of the female reproductive system of *S. litura*: A: corpus bursae; B: signum, C: ductus bursae; D: ostium bursae; and E: diverticulum of bursa copulatrix; F: ductis seminalis; G: spermathecal gland; H: utriculus I: lagena of spermatheca; J: ductus receptaculi; K: accessory gland (paired); L: accessory gland reservoir (paired); M: vestibulum; N: calyx of the unpaired oviductus communis; O: one of four ovarioles of ovary (paired); P: papillae anales; Q: rectum; X: corpus, Y: collum, and Z: frenum of spermatophores; (Source: Etman and Hooper, 1979).

3.2.1 Effects on ovarian development

Sublethal doses of chlorfluazuron (LD_{10}: 1.00 ng $larva^{-1}$; LD_{30}: 3.75 ng $larva^{-1}$), applied to newly ecdysed fifth-instar larvae significantly ($P<0.0001$) reduced the body weight, fresh

ovarian weight and dry ovarian weight in newly emerged adults when compared with the controls (Table 3). Significant reductions were not observed in fresh body weight (P=0.0567), fresh ovarian weight (P=0.7788) and dry ovarian weight (P=0.5757), when the LD_{10} and LD_{30} treatments were compared. Similarly, ratios of fresh ovarian/fresh body weight (31.0%), dry ovarian/fresh ovarian weight (28.0%) and dry ovarian/fresh body weight (9.0%), were not significantly different (Table 3) (Perveen and Miyata, 2000).

T[a]	n[a]	FBW[a,b] (M±SD) mg	FOW[a,b] (M±SD) mg	DOW[a,b] (M±SD) mg	% R=FOW /FBW[a,b] (M±SD)	% R=DOW /FBW[a,b] (M±SD)	% R=DOW /FOW[a,b] (M±SD)
C	30	255±11.6a	81.1±9.2a	23.6±1.4a	31.5±2.9a	28.9±2.4a	9.2±0.7a
LD_{10}	30	229±3.2b	72.0±3.4b	20.0±1.4b	31.2±1.5a	28.1±1.2a	9.0±0.8a
LD_{30}	30	224±5.9b	70.9±4.2b	19.9±1.3b	31.3±2.4a	28.0±0.5a	9.1±0.8a

[a]C: control; T: trearment; LD_{10}: 1.00 ng larva^{-1}; LD_{30}: 3.75 ng larva^{-1}; n: number of insects used; FBW: fresh body weight; FOW: fresh ovarian weight; DOW: dry ovarian weight; % R: percent ratio
[b]Data were analyzed using one-way ANOVA (Concepts, 1989) at P<0.001. Means within a column followed by different letters indicate significant differences according to Scheffe's F-test (Scheffe, 1953) at 5%.

Table 3. Effects of sublethal doses of chlorfluazuron on the ovarian and body weight of newly emerged adults after topical application to newly ecdysed fifth-instar larvae of *Spodoptera litura* (Source: Perveen and Miyata, 2000).

Ovaries are small on the 8th day after pupation. From the 8th day after pupation to the day before adult emergence, ovarian weight slowly increased; after that it increased sharply until the day of adult emergence, and then, increased gradually until the 2nd day after adult emergence, when it reached maximum (120±19.4 mg). Then, in the controls it decreased gradually until the 7th day after adult emergence. The pattern of changes in fresh ovarian weight in the LD_{10}- or LD_{30}-treated females was similar as observed in the controls during various developmental days of pupae and adults. The fresh ovarian weight was significantly reduced on the 8th day after pupation (P<0.0001); on the 9th day after pupation (P<0.0001); on the 1st day after adult emergence (P<0.0003); on the 2nd day after adult emergence (P<0.0001); on the 3rd day after adult emergence (P<0.0001); on the 4th day after adult emergence (P<0.0001); on the 5th day after adult emergence (P<0.0001); on the 6th day after adult emergence (P<0.0001); on the 7th day after adult emergence (P<0.0001) in the LD_{10}- or LD_{30}-treated females compared with the controls, but no significant reduction was observed (P=0.0979–0.970) between the LD_{10}- or LD_{30}-treated females during ovarian development (Figure 2) (Perveen and Miyata, 2000).

In newly emerged the LD_{10}- or LD_{30}-treated adults, the total length of the ovariole was significantly reduced (P<0.0001) compared with the control, but there were no significant reductions (P=0.0508) between the LD_{10}- or LD_{30}-treatments. In the LD_{10}- or LD_{30}-treated insects, the germarium (immature oögonia) was significantly longer (P<0.0001) than that of the pedicle (fully mature ova) and the vitellarium (under developing oöcytes) compared with the controls in which the vitellarium was significantly longer (P<0.0001) than the germarium and pedicle (Figure 3; Table 4) (Perveen and Miyata, 2000; Perveen, 2011).

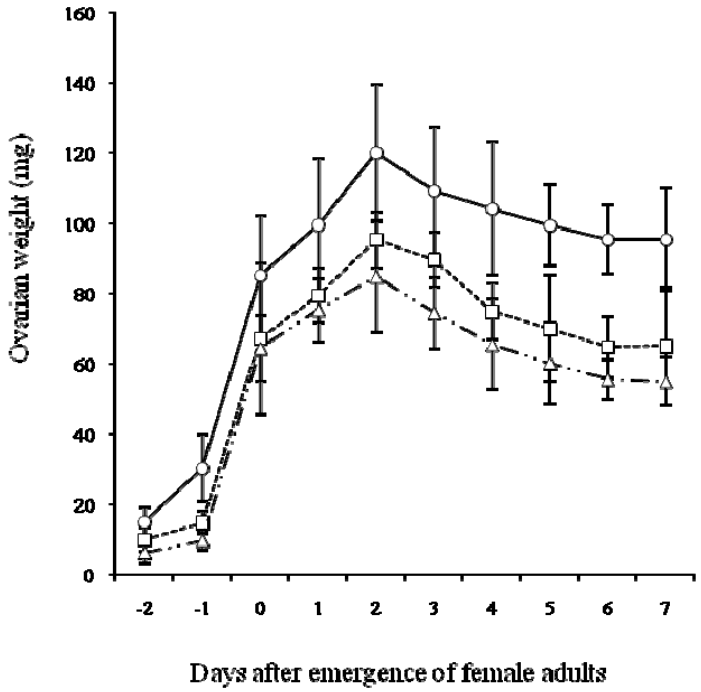

Fig. 2. Effect of sublethal doses of chlorfluazuron on ovarian weight during different developmental days (post pupal and 1 to 7 day after adult emergence). For control (○; n = 10), LD_{10} (1.00 ng larva^{-1}) treated (□; n = 9), and LD_{30} (3.75 ng larva^{-1}) treated (Δ; n = 9) after topical application to newly ecdysed fifth instars of *Spodoptera litura*. Data were analyzed using one-way ANOVA (Concepts, 1989) at $P<0.0001$ and Scheffe's *F*-test (Concepts, 1989) at 5%. Vertical bars indicate SD; (Source: Perveen and Miyata, 2000).

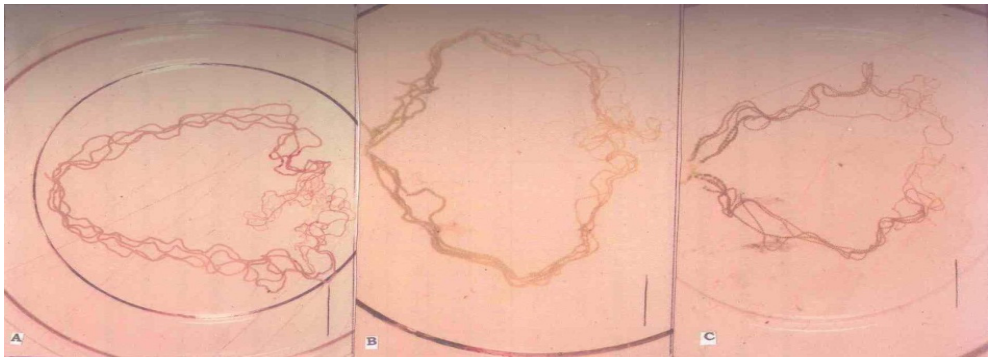

Fig. 3. A comparison of ovarian morphology of newly emerged adult *Spodoptera litura*, in A: untreated (control); B: treated with the LD_{10} dose and C: treated with the LD_{30} dose of chlorfluazuron. Bars in photographs indicate 100 µm (Source: Perveen, 2011).

Treat-ments[a]	n_1	n_2	TLO[bc] (M±SD)mm	LP[bc] (M±SD)mm	LV[bc] (M±SD)mm	LG [bc] (M±SD)mm	R P:V:G (M±SD)mm
Control	10	80	104.8±5.1a[3]	33.5±3.4a	45.2±4.4a	26.1±4.2a	32:43:25
LD_{10}	9	72	91.5±5.5b	22.1±5.0b	30.5±2.1b	39.0±2.7b	24:33:43
LD_{30}	9	72	88.7±9.6b	20.4±4.4	28.6±6.7b	40.0±8.9b	23:32:45

[a]LD_{10}, 1.00 ng larva^{-1}; LD_{30}, 3.75 ng larva^{-1}; n_1: number of insects used; n_2: number of ovariole measured; TLO: total length of ovariols; LP: length of pedicle; LV: length of vitellarium; LG: length of germarium; RP:V:G: Ratio of pedicle:vitellarium:germanium

[b]In Spodoptera litura, the paired ovaries are composed of 8 ovarioles. Four ovarioles are found on each side of the body cavity, forming several loops. Each ovarioles differentiated into 3 parts: (1) pedicle (fully matured eggs), (2) vitellarium (oöcytes and trophocytes), (3) germanium (oögonia) (Etman and Hooper 1979).

[c]Data were analyzed using one-way ANOVA (Concepts, 1989) at $P<0.0001$. Means within a column followed by different letters indicate significant differences according to Scheffe's F-test (Scheffe, 1953) at 5%.

Table 4. Effects of sublethal doses of chlorfluazuron on ovarian development in newly emerged adults after topical application to newly ecdysed fifth instars of *Spodoptera litura* (Source: Perveen and Miyata, 2000).

When ratios of the length of the pedicle, vitellarium and germarium were compared, they were 32:43:25 for the controls, 24:33:43 for the LD_{10} and 23:32:45 for the LD_{30}. There was a significant reduction when the %ratios of the LD_{10} and LD_{30} were compared with the controls, but there was significant reduction between the LD_{10} and LD_{30} treatments (Table 4). When ovarian maturation was observed untreated females had mature ova with an occasional one or two being absorbed (solid ova) in the ovarioles. In the LD_{10}-treated females, the spacing in the ovarioles and the absorption of ova different from the control. In LD_{30}-treated females, besides the spacing and absorption , sometimes only immature ova (germarium) were found in the ovarioles (data is not presented) (Perveen and Miyata, 2000). Mature ova were not observed in the pupae during the 2nd day before adult emergence, but a few mature ova were found the day before adult emergence. The number of mature ova sharply increased until the 1st day after adult emergence, and then gradually increased until the 2nd day after adult emergence. The maximum number of mature ova 725±2.0 was found on the second day after adult emergence. On the same day, the number of mature eggs was significantly reduced ($P<0.0001$) in the LD_{10}- or LD_{30}-treated females as compared with the controls, but no significant reduction was observed ($P=0.0984$) between the LD_{10} and LD_{30} treatments. From the 2nd day to the 7th day after adult emergence, absorption of mature ova started gradually in the controls, and the LD_{10}- or LD_{30}-treated females. The pattern of maturation of ova in ovaries was similar in the controls, LD_{10} and LD_{30} treatments (Figure 4) (Perveen and Miyata, 2000).

The cell density, expressed as number of nuclei per mm^2, was determined at various days during sexual maturation in the germaria of the controls, LD_{10}- or LD_{30}-treated females (Table 4.3). In the controls, on the 2nd day of adult emergence, the density was 1636±9.17 nuclei mm$^{2-1}$. The cell density increased until the 3rd day, when it was 1829±8.87 nuclei mm$^{2-1}$ and decreased thereafter. On the 4th day of adult emergence, the cell density was 1323±56.20 nuclei mm$^{2-1}$. In the LD_{10}- or LD_{30}-treated insects, the patterns of the cell density change in the germarium were the same as in the controls but the values were significantly ($P<0.01$) lower in the LD_{10}-treated females and more were significantly decreased in the LD_{30}-treated females compared with the controls, during the days the 2nd, 3rd and 4th of adult female development. There

was also a significant reduction ($P<0.05$) in the cell density between the LD_{10}- and LD_{30}-treated females during adult development (Table 5) (Perveen, 2011).

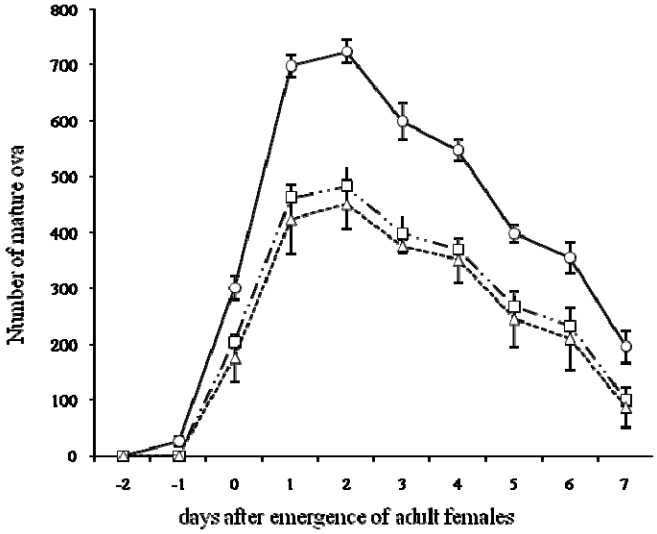

Fig. 4. Effect of sublethal doses of chlorfluazuron on number of mature eggs in the ovaries during different developmental days (post pupal and 1 to 7 day after adult emergence). For control (○; n = 13), LD_{10} (1.00 ng larva^{-1}) treated (□; n = 11), and LD_{30} (3.75 ng larva^{-1}) treated (Δ; n = 13) after topical application to newly molted fifth instars of *Spodoptera litura*. Data were analyzed using one-way ANOVA (Concepts, 1989) at $P<0.0001$ and Scheffe's *F*-test (Scheffe, 1953) at 5%. Vertical bars indicate SD; (Source: Perveen and Miyata, 2000).

			Cell density number of nuclei (mm^2)$^{-1}$ in the germarium during female adults age (M±SD)[b,c]		
Treatments[a]	n_1	n_2	2 day-old	3 day-old	4 day-old
Control	5	10	1636±9.17a	1829±8.87a	1323±56.20a
LD_{10}	5	10	1570±42.50b	1753±49.91b	1235±9.50b
LD_{30}	5	10	1489±8.60c	1644±7.68c	1089±61.42c

[a]LD_{10}: 1.00 ng larva^{-1}; LD_{30}: 3.75 ng larva^{-1}; n_1: number of insects used; n_2: number of ovariole measured

[b]The age of female adults was taken from the day of adult emergence.

[c]Data were analyzed using one-way ANOVA (Concepts, 1989) at $P<0.01$. Means within a column followed by different letters indicate significant differences according to Scheffe's F-test (Scheffe, 1953) at 5%.

Table 5. Effects of sublethal doses of chlorfluazuron on the cell density in the germarium after topical application to newly ecdysed fifth-instar larvae of *Spodoptera litura* (Source: Perveen, 2011).

3.2.2 Effects on oöcytes development

In the controls, the basal oöcytes were tiny on the 5th day after pupation, but increased sharply until the 8th day, after which they increased slowly until adult emergence. The maximum size of the basal oöcytes on the day of adult emergence was significantly reduced ($P<0.0002$) in the LD_{10}- or LD_{30}-treated females, but there was no significant difference ($P=0.9976$) between LD_{10}- and LD_{30}-treated females (Figure 5) (Perveen and Miyata, 2000).

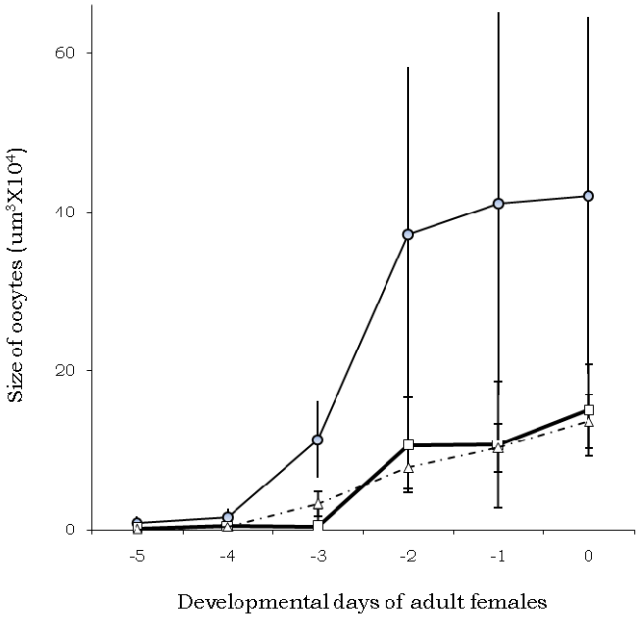

Fig. 5. Effect of sublethal doses of chlorfluazuron on size of basal oöcytes during different developmental days (5 to 9 day after pupation pupae and newly emerged adults). For control (○), LD_{10} (1.00 ng larva^{-1}) treated (□), and LD_{30} (3.75 ng larva^{-1}) treated (Δ) after topical application to newly ecdysed 5th instars of *Spodoptera litura*. Data were analyzed using one-way ANOVA (Concepts, 1989) at $P<0.0002$ and Scheffe's *F*-test (Scheffe, 1953) at 5%. Verticle bars indicate SD (n = 9-10); (source: Perveen and Miyata, 2000).

The thickness of the follicular epithelium of the basal oöcytes gradually increased and reached a maximum on the 8th day after pupation, after which it sharply declined. On the 8th day after pupation, it was significantly reduced ($P<0.005$) in the LD_{10}- or LD_{30}-treated females when compared with control females, but there was no significant difference ($P=0.8686$) between the LD_{10} or LD_{30} treatments. The reduction was approximately 34% in the LD_{10}- and 39% in the LD_{30}-treated females. The patterns of the development of the follicular epithelium of basal oöcytes were not similar in the LD_{10}- or LD_{30}-treated females compared with the controls. However, pattern was similar between the LD_{10}- and LD_{30}-treated females. In the LD_{10}- or LD_{30}-treated females, the follicular epithelium reached maximum on the 9th day after pupation, and then declined. The development of the follicular epithelium was delayed by one day in the LD_{10}- or LD_{30}-treated females compared with the controls. On the 9th day, it was thicker in the LD_{30}-treated females and thickest in

LD_{10}-treated females than in control females, but significant differences were not observed (P=0.7611) among these three groups, i.e. controls, LD_{10}- and LD_{30}-treated females (Figure 6) (Perveen and Miyata, 2000).

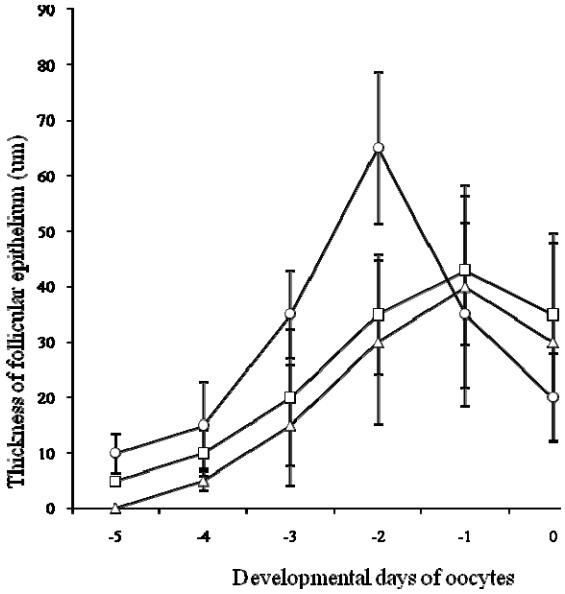

Fig. 6. Effect of sublethal doses of chlorfluazuron on thickness of follicular epithelium of basal oöcytes during different developmental days (5 to 9 days after pupation and newly emerged adults). For control (○), LD_{10} (1.00 ng larva^{-1}) treated (□), and LD_{30} (3.75 ng larva^{-1}) treated (Δ) after topical application to newly ecdysed 5th instar larvae of *Spodoptera litura*. Data were analyzed using one-way ANOVA (Concepts, 1989) at $P<0.005$ and Scheffe's *F*-test (Scheffe, 1953) at 5%. Vertical bars indicate SD (n = 9-10); (Source: Perveen and Miyata, 2000).

3.3 Discussion

Topical application of sublethal doses of chlorfluazuron (LD_{10}: 1.00 ng per larva or LD_{30}: 3.75 ng per larva) on newly ecdysed fifth instars had an effect on the reproduction of *S. litura* by reducing fecundity, fertility, and hatchability (Perveen, 2000a). Thus, this study was conducted to establish the causes of the reduction in these parameters. It was found that topical appication of sublethal doses of chlorfuazuron had an affect on ovarian development and oögenesis by decreasing the weight of ovaries during postpupal and adult developmental days of LD_{10} or LD_{30} treated females. The basic factors responsible for the reduction in ovarian weight were reduction in the length of different parts of the ovarioles, decrease in the number of mature ova, reduction in the size of basal oöcytes and thickness of their follicular epithelium, and reduction in protein content of ovarian constituents as compared with the controls. In this study, topical application of sublethal doses of chlorfuazuron signifcantly reduced the ovarian weight to that of the controls in postpupal and adult developmental days (Figure 2; Table 4.3). The ratios of fresh ovarian/fresh body

weight, dry ovarian/fresh ovarian weight, and dry ovarian/fresh body weight were the same among the newly emerged control, LD_{10} or LD_{30} treated females, indicating a reduction in the body, fresh, and dry ovarian weights with the same degree of reduction in treated females and controls. However, Soltani and Pickens and DeMilo (1977) reported that 0.5 mg DFB, when topically applied at pupal ecdysis to *Cydia pomonella* (L.), did not cause the ovarian weight be significantly reduced (P<0.05) between control and treated newly emerged adult females. Nor did Hatakoshi (1992) observe any signifcant reduction (P<0.05) in the ovarian weight between control and treated last day of pupae to third day after adult emergence of *S. litura,* when 0.3 ng pyriproxyfen was topically applied at pupal ecdysis. The differences in results observed in these experiments may be related to the pesticides, the kind of insect used and their developmental stages. *Spodoptera litura* has paired ovaries that branch into four polytrophic meroistic ovarioles located on the ventral side of the body cavity, making several loops of ovarioles, with all basal oöcytes developing simultaneously each ovariole is differentiated into tree portions according to the developmental stages of the oöcytes: (1) the yellowish green pedicle, where fully matured ova are stored; (2) the reddish orange vitellarium, which contains the developing oöcyte and trophocyte follicles which undergo accumulation of yolk proteins, and choriogenesis; and (3) the whitis germarium, which contains oögoia, from which germ cells proliferate and follicles are formed. Similar observations were reported by Riakhel and Dhadialla (1992) and Etman and Hooper (1979), which were confirmed here.

As in other Lepidopterous species, the ovaries of *S. litura* start to differentiate, and develop at the pupal stage. Indeed, in controls, the thickness of follicular epithelium of basal oöcyte reached its maximum size on the eighth day after pupation. This coincided with the start of follicular epithelium resorption. Histological examination on *S. litura* showed that topical application of sublethal doses of chlorfuazuron to newly ecdysed ffth-instar larvae affected growth and development of oöcytes during pupal and adult stages by affecting size and thickness of follicular epithelium (Figures 5 and 6). However, in *C. pomonella,* (in controls) the basal oöcytes reached their maximum size 7 day after pupation. In this insect, this coincided with the start of follicular epithelium resorption. Hence, a 0.5-mg dose of DFB applied topically to newly ecdysed pupae affected the growth and development of oöcytes by causing a decrease in both the thickness of the follicular epithelium and size of basal oöcytes during the pupal development (Soltani and Mazouni, 1992). Lim and Lee (1982) reported that 2-d-old adult females of *O. japonica,* starved for 6 h and consumed 500 mg (AI) of DFB with two maize discs. The females were found to have retarded ovarian development, caused by a delay of oöcytes development, and an increased percentage of oöcytes resorption. This caused a decrease in fecundity and egg viability of the females. However, significant reduction was not observed either in the number of ovarioles or in the length of basal oöcytes in treated insects. Differences in these results might be a result of the use of different BPUs. Also, the doses used by Lim and Lee (1982) were very high compared with those used in present study or in the Soltani and Mazouni (1992) experiments.

In newly emerged treated adults, the germarium was much longer than the pedicle and vitelarium as compared with the controls in which the vitelarium was longer than the germarium and pedicle (Table 4). This shows that maturation of oöcytes was delayed in treated adult females as compared with the controls. The maximum thickness of the follicular epithelium of basal ooctes was observed on the 9 day after pupation in treated females, whereas it was on the 8 d after pupation in the controls (Figure 6). Subsequent to the 8th or 9th day, resorption of follicular epithelium started in control and treated females,

respectively. When ovarian maturation was scored, as depicted in Figure 4.4, a maximum number of matured oöcytes were found in the second day after adult emergence in the controls. From this day, resorption of mature oöcytes started. The chlorfuazuron-treated females showed the same pattern of mature oöcyte resorption up to the seventh day after adult emergence as in the controls. However, Hatakoshi (1992) reported that when 0-day pupae of female *S. litura* were topically treated with pyriproxyen (0.3 ng per pupa), few or no mature oöcytes were found in newly emerged females, but controls had mature oöcytes with one occasionally being resorbed. The maturation of insect eggs dependent, among other factors, on the materials taken up from the surrounding hemolymph (Telfer et al., 1981), and by materials synthesized by the ovary in situ (Indrasith et al.,1988). These materials include proteins, lipids, and carbohydrates, all of which are required for the embryogenesis (Kunkel and Nordin 1985, *Kanost* et al., 1990). Difubenzuron also caued a decrease in ovarian protein content in *C. pomonella* (Soltani and Mazouni, 1992). Decrease in the ovarian protein content suggests an interference of BPUs with vitellogenesis. It has been reported that DFB could affect ecdysteroid secretion from other organs, such as the epidermis, in *T. molitor* (Soltani, 1984), ovaries in *C. pomonella* (Soltani et al., 1989a; 1989b), and the concentration of hemolymph constituents in *T. molitor* (Soltani, 1990). Future studies should clarify the biochemical mechanism. Moreover, this work dose not clarify why signiÞcant differences were not observed (P<0.0001) between effects of LD_{10} (1.00 ng larva^{-1}) and LD_{30} (3.75 ng larva^{-1}) treated females, although LD_{30} dose was much higher than LD_{10} dose. Further studies are needed to obtain more knowledge about the effects of chlorfuazuron on oögenesis. Currently, the biochemical mechanism involved has been explored.

3.4 Conclusion

Sublethal doses of chlorfiuazuron (LD_{10}: 1.00 ng larva^{-1} or LD_{30}: 3.75 ng larva^{-1}) topically applied on newly ecdysed fifth instars of *S. litura* significantly reduced ovarian weight and number of mature eggs in pupae and adults, compared with those of the controls. The ratios of fresh ovarian/fresh body weight, dry ovarian/fresh ovarian weight, and dry ovarian/fresh body weight were the same among controls, LD_{10}, and LD_{30} treated newly emerged adults. In treated adults, the germarium was significantly longer than the pedicle and vitelarium compared with those of the controls, whereas in controls the vitelarium was significantly longer than the germarium and pedicle. This indicates a delayed of maturation of ovarioles in treated cutworms. These doses also disrupt growth and development of oöcytes by significantly affecting the size of basal oöcytes and thickness of follicular epithelium. The maximum size of basal oöcytes recorded on the day of adult emergence was significantly reduced in LD_{10} or LD_{30} treated females, compared with those of the controls. The thickness of the follicular epithelium of basal oöcytes reached to a maximum in the controls on the 8th day and in treated females on the ninth day after pupation. The effects of chlorfiuazuron on ovarian development and oögenesis are presumed to be responsible for the reduction in fecundity caused by sublethal exposure to chlorfiuazuron.

4. Effects of sublethal doses of chlorfluazurn on male reproductive system of *Spodoptera litura*

The deep yellow-coloured testes of *S. litura* are distinctly paired in larvae and they appear as a single round organ in adults. The testes of *S. litura* resemble those of other lepidopterans,

being enclosed in a common membrane called the scrotum. The testes lie dorsally and appear to be held in place by trachea and strands of basement membrane-like material (Amaldoss, 1989). Although reports of several layers surround the testes of lepidopteran have been made, a single capsule and follicular layers are present in *S. litura* (Amaldoss, 1989). Chase and Gilliland (1972) described the *tunica externa* and *interna* as nothing more than basement membranes over the capsule and follicular layers. The intra-follicular layer is divided into eight incomplete compartments in the tobacco leafminer, *Phthorimaea operculella* (Zeller). This layer is similar to that in the larger canna leafroller, *Calpodes ethlius* (Stoll), and tobacco moth, *Ephestia elutella* (Hübner). It also bears the pigments responsible for the bright yellow-coloured testes. It is clear that spermatogenesis persists in the adult testis.

A characteristic feature of the testicular follicles is the presence of large cells or a nucleated mass of protoplasm in the apex of the germarium. This is known as an apical cell or versonian cell. This is the region where there are successive stages of development of the germ cells occur. The upper part contains the primary spermatogonia and is known as the germarium. This is followed by a region called the zone of growth. The region or zone of growth is where spermatogonia multiply and usually become encysted. The maturation zone, where maturation takes place follows. Finally, is the zone of transformation where the spermatocytes develop into spermatids (spermiogenasis) completing spermatogenesis (Amaldoss, 1989). Two distinct types of spermatozoa are produced in the Lepidoptera: eupyrene (nucleated) spermatozoa which can fertilize the egg; and apyrene (anucleated) which are smaller and completely lacking in nuclear material, and do not appear to play any role in activation of the eggs (Doncaster, 1911; Goldschmidt, 1916). The eupyrene sperm can easily be counted in the male tract because they remain in bundles until they are transferred during mating, but the apyrene sperm are dispersed shortly after they leave the testis. Like eupyrene sperm, the apyrene sperm are produced in large numbers, usually contributing over half the sperm complement, and are transferred to the females with the eupyrene sperm during mating. It was thought that the apyrene sperm did not appear to play any role in activation of the eggs (Friedlander and Gitay, 1972). Their function has remained unclear ever since their discovery by Meves (1902), although several hypotheses concerning the function of the apyrene sperm have been proposed (Silberglied et al., 1984). Holt and North (1970 b) proposed that, in the cabbage looper, *Trichoplusia ni* (Hüebner), apyrene sperm might aid the transport of eupyrene sperm from the male reproductive tract to the female reproductive tract. Katsuno (1977 a) reported that the apyrene sperm in the *B. mori*, might facilitate the migration of eupyrene sperm through the cellular barrier, separating the testis from the efferent ducts. Gage and Cook (1994) reported that nutritional stress seriously affected the number and size of eupyrene and apyrene sperm production in the Indian meal moth, *Plodia interpunctella* (Hübner). However, sperm development in Lepidoptera takes place in the larvae (Munson, 1906; Machida, 1929; Garbini and Imberski, 1977). Usually, spermatogenesis starts in the late larval instars and proceeds on a schedule well correlated with the insect's metamorphosis. Studies *in vitro* and *in vivo* indicated that high titre of juvenile hormone inhibits spermatogenesis, and that sperm mitosis and meiosis require sufficient ecdysteroid titre. During the post-embryonic development of eupyrene and apyrene sperm bundles, when the insect is going to pupation, the juvenile hormone titre declines (Leviatan and Friedlander, 1979). Other factors have also been reported to promote spermatogenesis *in vitro* and *in vivo* (Dumser, 1980 a). In adult males *S. litura*, both apyrene and eupyrene sperm appear in bundles in the testis, but in the vas deferens only the eupyrene sperm were still in bundles, as reported for *B. mori* (Katsuno 1977 b), *T. ni* (Holt

and North, 1970 a) and the army worm, *Pseudaletia separata* (Walk.) (He, 1994). The testes of early larvae contain a large number of spermatogonial cells near the outer border of the follicles. There is a preponderance of spermatocytes containing primary spermatocytes during the penultimate and early last-instar larvae. Secondary spermatocytes are present in the early last-instar larvae, persisting through to the middle of the last-instar larvae. They then begin to differentiate into spermatids. In the process of elongation and maturation of spermatids, the spermatocytes assume an elliptical shape. The sperm bundles formed as a result of maturation of the spermatids are seen abundantly in adults. Spermiogenesis is, however, not synchronous, and spermatozoa in various stages of differentiation can be detected in the testes of freshly emerged adults (Sridevi et al., 1989a). In the pupa, the apyrene sperm bundles emerge from the testicular follicle into the vas efferens earlier than the eupyrene sperm bundles and the bundles separate when they pass through the basement membrane of the testis (Katsuno, 1977b). The apyrene spermatozoa migrate from the vas efferens into the seminal vesicle through the vas deferens during the pupa (Katsuno, 1977c). In the post-pupal period, however, the eupyrene sperm bundles and apyrene spermatozoa migrate simultaneously through the same way (Katsuno, 1977d). The effects of chlorfluazuron have been examined on male reproductive system during testicular development and spermatogenesis when sublethal doses have been topically applied to newly ecdysed fifth-instar larvae of *S. litura*.

4.1 Experimental procedure

4.1.1 Histology of testis

Testes from newly molted sixth instar larvae to 5-day-old virgin adult males (treated and control), were dissected in 0.9% of NaCl under a binocular microscope. The length and width of each testis were measured by the same procedure as used for the oöcytes measurement. Testis volume was calculated for larval testes using the formula $4/3\pi$ (length×width^2), assuming that the testis is a prolate spheroid (Loeb et al., 1984). For the fused pupal and adult testes, the formula $4/3\pi r^3$ was used, with r as the radius of the globular gonad. The treated and control weight and sheaths thickness of testes were measured by the same procedure used for the ovaries as described above (Perveen, 2000b).
The thickness of treated and control testes sheaths or vas deferenti of untreated and treated relevant stages of insect was observed by making a parafilm microtomy conducted according to the method used by Yoshida (1994) and the procedure to stain the nuclei of sperm was adapted from the method by He (1994) (Perveen, 2000b).

4.1.2 Spermatogenesis

A staining method was used for determining number of the cysts, eupyrene and apyrene sperm for treated and control (He et al., 1995). First, the testis was transferred to a microscopic grid slide (each square= 1mm^2) and crushed until it was evenly distributed on the slide. Secondly, several drops of methanol-acetic acid solution (3 : 1; v/v) were added to the slide to fix the reparation for 15 min, and the excess fixing solution was absorbed with filter paper. Third, several drops of 2-5% Giemsa solution dissolved in Sorensen-Gomori buffer solution (monobasic and dibasic sodium phosphate, 0.07 M, pH 6.8) were added to the slide to stain the preparation for 10-30 min. The slide was washed with water and air dried after staining. Finally, the air-dried preparation was observed for counting of bundles and cysts under a phase contrast microscope at 20×mignification. Cysts were classified into the following six developmental stages as described by Chaudhury and Raun (1966): (1)

spermatogonia; (2) primary spermatocytes; (3) secondary spermatocytes; (4) spermatids; (5) elongated cysts with maturing sperm and (6) bundles with fully matured sperm. The length and width of sperm bundles were measured with a calibrated ocular micrometer a phase contrast microscope at 400×magnification (Perveen, 2000b).

4.1.3 Data analysis

Data were analyzed using analysis of variance, one way ANOVA (Concepts, 1989) at $P<0.0001$ and Scheffe's F-test (Scheffe, 1953) at 5%.

4.2 Results

The structural morphogenesis was seen in sixth-instar larvae of *S. litura* during development. When fifth-instar larvae ecdysed into newly sixth-instar larvae (N: 0 day), larvae remained unchanged for upto 2 hour. Then, they changed to a slender surface during 1st day (S). After that, they changed to being puffy during 2nd day (early-last-instar stage: P). Then, they changed to digging stage during 3rd day (mid-last-instar stage: D). Then, they changed to early burrow during 4th day (pre-late-last-instar stage: B_1). After that, it changed to late burrow during 5th day (post-late-last-instar stage: B_2). The morphogenesis for phase variations used for convenient of observations during experiments is given in Table 6. The morphology of the adult male reproductive system of *S. litura* is shown in Figure 7 (Perveen, 2000b).

Developmental category (days)	Symbols for phase variations[a]	Name of the phases[a]	Duration (hour day^{-1})
0	N	newly ecdysed	0–2
1	S	slender surface	1st
2	P	puffy (early last instar stage)	2nd
3	D	digging (mid last instar stage)	3rd
4	B_1	early burrowed (pre late last instar stage)	4th
5	B_2	late burrowed (post late last instar stage)	5th

[a]Symbols for phase variations observed during developmental days of sixth-instar larvae were used for the convenience of observations.

Table 6. Structural morphogenesis during five developmental days of sixth-instar larvae of *Spodoptera litura* (Source: Perveen, 2005).

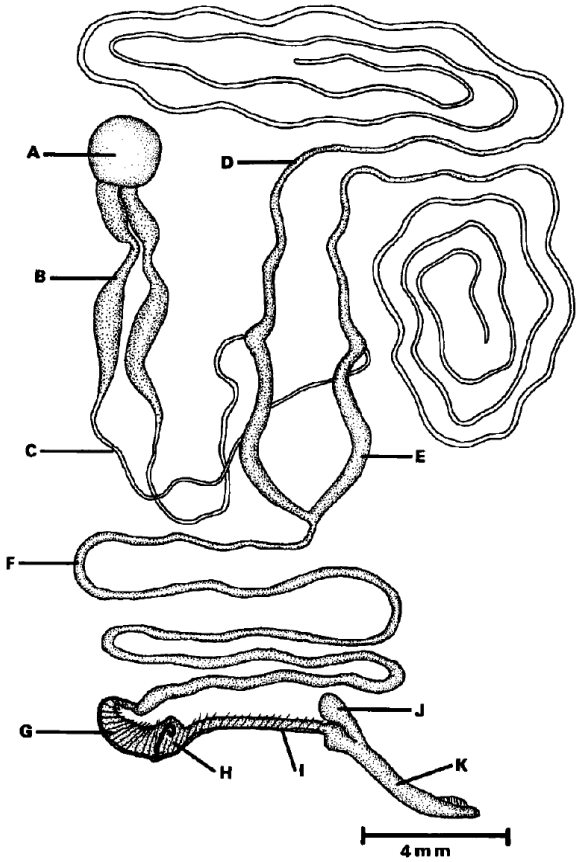

Fig. 7. The morphology of the male reproductive system of *Spodoptera litura*: A: testis; B: seminal vesicle (paired); C: vas deferens (paired); D: accessory glands (paired); E: ductus ejaculatorious duplex; F: primary segment of ductus ejaculatorious simplex; G: muascular area; H: area of frenum formation; and I: area of collum formation of the cuticular secondary segment of the ductus ejaculatorious simplex; J: caecum of aedeagus; K: aedeagus (Source: Etman and Hooper, 1979).

4.2.1 Effects on testicular development

The testes of *S. litura* show the three dimentional measureable structure. Each mature testis consists of four follicles or lobes, each separated by an inner layer of sheath cells. An outer sheath cell layer further surrounds all follicles. In sixth-instar larvae, the volume and weight of the testes gradually then rather sharply increased until the 4th day of sixth-instar larvae. The volume weight decreased when two larval testes fused on the 5th day (last day) after moulting of sixth-instar larvae. They again sharply increased in size, reached a maximum (6.21±1.31 mm^3 and 11.94±0.42 mg, n=30, respectively) on the 0 day of pupation and gradually declined until the 5th day after adult emergence. Sublethal doses of chlorfluazuron rapidly disrupted the development of testes by decreasing the volume and weight of testes

compared with the controls. The weight and size of testes were significantly reduced ($P<0.001$) in the LD_{10}-treated and more significantly reduced ($P<0.0001$) in LD_{30}-treated males compared with the controls from newly ecdysed sixth-instar larvae to the 5th day after adult emergence. Testes reached their maximum size in treated males (LD_{10}: 4.16±1.54 mm^3 and 8.0±0.83 mg; LD_{30}: 2.79±1.00 mm^3 and 4.88±1.05 mg; n= 10, respectively) on the same day as the controls. The patterns of development of the testes with respect to the volume and weight were the similar in the controls and the LD_{10}- or LD_{30}-treated males (Figures 8a and b) (Perveen, 2000b).

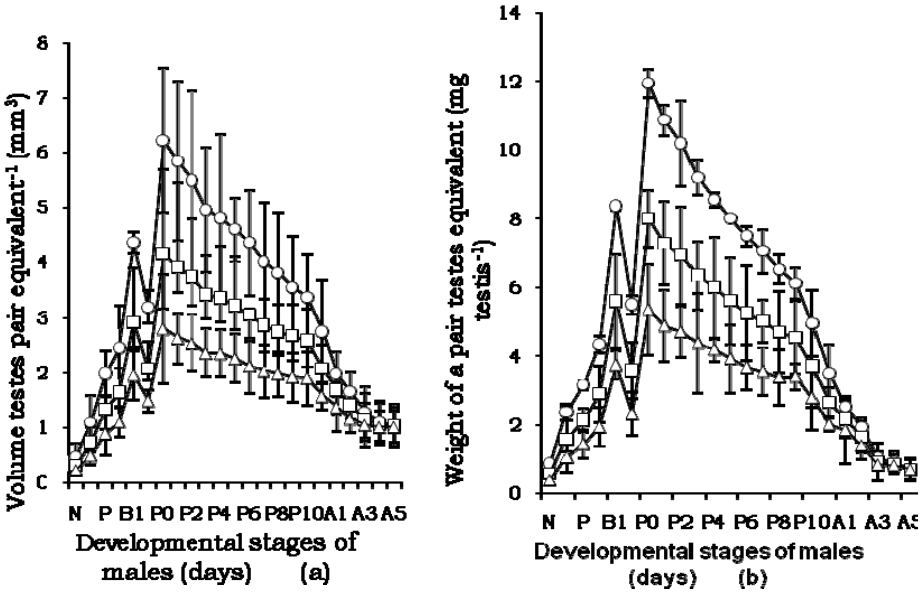

Fig. 8. Effects of sublethal doses of chlorfluazuron (LD_{10}: 1.00 ng $larva^{-1}$; LD_{30}: 3.75 ng $larva^{-1}$) on the testis volume (a) and weight (b) of *Spodoptera litura* during newly ecdysed sixth-instar larvae to 5th day after adult emergence; controls: O; LD_{10}: □; LD_{30}: Δ; data analyzed using one-way ANOVA (Concepts, 1989) at $P<0.0001$ and Scheffe`s *F*-test (Scheffe, 1953) at 5%; vertical bars: SD; N, S, P and D: larval (Table 5.1), P: pupal and A: adult developmental days; n = 10 for each point; paired larval testes and fused single pupal or adult testis were considered as testes pair equivalent; (Source: Perveen, 2000b).

The thickness of the testis sheath gradually and then sharply increased until the 4th day of moulting of sixth-instar larvae. It decreased when the two larval testes fused on the 5th day (last-day) of the sixth-instar larvae; it again increased and reached to a maximum [(6±0.51)×10^{-2} mm; n=10] on the 0 day of pupation and gradually declined in the newly emerged adults. The thickness remained constant until the 2nd day after adult emergence. Sublethal doses rapidly disrupted the development of testis by significantly decreasing ($P<0.0001$) the thickness of the testes sheath as compared with that of the controls. This reduction occurred from newly ecdysed sixth-instar larvae to the 0 day of pupation in the LD_{10}-treated and the 1st day after pupation in the LD_{30}-treated males. The thickness of the

testes sheath in chlorfluazuron-treated males reached a maximum [(5.9±0.67)×10^{-2} mm] in the LD_{10}-treated males on the 1st day and [(5.9±1.1)×10^{-2} mm] in the LD_{30}-treated males on the 2nd day after pupation whereas, in the controls, it was on the 0 day of pupation. This result shows that attainment of the maximum thickness of the testes sheath was delayed by one day in LD_{10}- and by two days in LD_{30}-treated males compared with the controls. However, no significant reduction was observed in the maximum thickness of the testes sheath among the control and LD_{10}- or LD_{30}-treated males. The developmental pattern of the testes sheath in the LD_{10}- or LD_{30}-treated males was similar to that of the controls (Figure 9) (Perveen, 2000b).

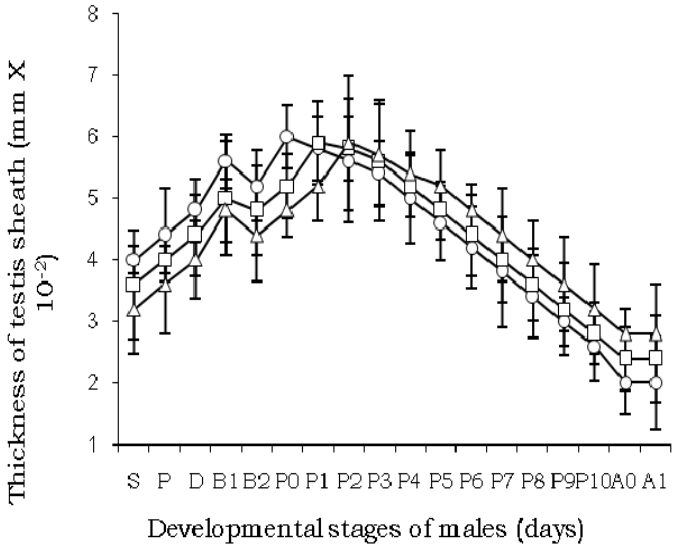

Fig. 9. Effects of sublethal doses of chlorfluazuron (LD_{10}: 1.00 ng larva^{-1}; LD_{30}: 3.75 ng larva^{-1}) on the thickness of testis sheath of *Spodoptera litura* during newly ecdysed sixth-instar larvae to 5th day after adult emergence; controls: O; LD_{10}: □; LD_{30}: Δ; data analyzed using one-way ANOVA (Concepts, 1989) at $P<0.0001$ and Scheffe`s *F*-test (Scheffe, 1953) at 5%; vertical bars: SD; S, P, D and B_1: larval (Table 5.1), P: pupal and A: adult developmental days; n = 10 for each point; paired larval testes and fused single pupal or adult testis were considered as a testes pair equivalent; (Source: Perveen, 2000b).

4.2.2 Effects on spermatogenesis

When the different developmental stages of cysts were observed in testes during spermatogenesis on the 1, 3 and 5 day-old sixth-instar larvae, the number of spermatogonia, primary and secondary spermatocytes was significantly reduced ($P<0.001$) in the LD_{10}- and even more significantly reduced ($P<0.0001$) in the LD_{30}-treated males as compared with those of controls. Spermatids, elongated cysts with mature sperm and eupyrene sperm bundles were not found in the controls and LD_{10}- or LD_{30}-treated larval testes (Table 7) (Perveen, 2000b).

TS[a]	T[a]	n[a]	*Sg[b] (M±SD)	*PS[b] (M±SD)	*SS[b] (M±SD)	St (M±SD)	ECMS (M±SD)	ESB (M±SD)
1st	C	13	2447±18a	4893±35a	1397±18a	0.0±0.0	0.0±0.0	0.0±0.0
	LD_{10}	11	1960±7b	3920±7b	1120±6b	0.0±0.0	0.0±0.0	0.0±0.0
	LD_{30}	10	1320±6c	2641±5b	754±4c	0.0±0.0	0.0±0.0	0.0±0.0
3rd	C	13	1311±8a	4280±9a	3146±8a	0.0±0.0	0.0±0.0	0.0±0.0
	LD_{10}	11	1050±5b	3430±7b	2520±7b	0.0±0.0	0.0±0.0	0.0±0.0
	LD_{30}	10	707±5c	2311±7c	1689±7c	0.0±0.0	0.0±0.0	0.0±0.0
5th	C	13	437±8a	3574±5a	4717±7a	0.0±0.0	0.0±0.0	0.0±0.0
	LD_{10}	11	342±5b	2870±7b	3780±6b	0.0±0.0	0.0±0.0	0.0±0.0
	LD_{30}	10	224±6c	1933±8c	2546±9c	0.0±0.0	0.0±0.0	0.0±0.0

[a]TS: treatment stages; T: treatments; n: number of males used; C: control; LD_{10}: 1.00 ng larva^{-1}; LD_{30}: 3.75 ng larva^{-1}; Sg: spermatogonia; PS: primary spermatocytes; SS: secondary spermatocytes; St: spermatids; ECMS: elongated cysts with mature sperm; ESB: eupyrene sperm bundles; larvae ecdysed usually during 0200 to 0800 hour and collected between 0800 to 1000 hour

[b]Data were analyzed using one-way ANOVA (Concepts, 1989) at $P<0.0001$. Means within columns followed by different letters are significantly different by Scheffe`s F-test (Scheffe, 1953) at 5%.

Table 7. Effect of sublethal doses of chlorfluazuron on spermatogenesis in the testes of sixth-instar larvae during 1st, 3rd and 5th days of development after topical application to newly ecdysed fifth-instar larvae of *Spodoptera litura* (Source: Perveen, 2000b).

When different developmental stages of the cysts were observed in testis during spermatogenesis in newly ecdysed pupae, on the 5th and 10th day after pupation, the number of spermatogonia, primary and secondary spermatocytes, were decreased. However, the spermatids and elongated cysts with mature sperm gradually increased in controls. Eupyrene sperm bundles were not present on the 0-5 day-old pupae. However, they were found on the 10th day after pupation (mean: 1002±3.0 numbers). The pattern of spermatogenesis was the same in the controls, LD_{10}- and LD_{30}-treated male pupae. However, the developmental stages of the sperm were significantly reduced ($P<0.001$) in the LD_{10}-treated and even more significantly reduced ($P<0.0001$) in the LD_{30}-treated males compared with the controls (Table 8) (Perveen, 2000b).

Different developmental stages of cysts were observed in testes during spermatogenesis in newly emerged, 1 and 2 day-old adults. The spermatogonia were not present in newly emerged and 1 day-old adults. Primary spermatocytes were not found in 1 and 2 day-old adults, but they (mean: 174±4.0 number) were found in newly emerged adults. The secondary spermatocytes, spermatids and elongated cysts with mature sperm were present, but gradually decreased in number in the controls. Eupyrene sperm bundles gradually increased in number in the controls. The pattern of spermatogenesis was the same in the controls, LD_{10}- and LD_{30}-treated male pupae. However, the stages of sperm development were significantly reduced ($P<0.001$) in the LD_{10}- and even more significantly reduced ($P<0.0001$) in LD_{30}-treated males as compared with the controls (Table 9) (Perveen, 2000b).

TS[a]	T[a]	n[a]	Sg[b] (M±SD)	PS[b] (M±SD)	SS[b] (M±SD)	St (M±SD)	ECMS (M±SD)	ESB (M±SD)
1st	C	13	262±6a[c]	1747±5a	2710±7a	3670±6a	349±8a	0.0±0.0
	LD_{10}	11	205±6b	1400±4b	2170±6b	2940±7b	280±5b	0.0±0.0
	LD_{30}	10	142±5c	943±6b	1462±5c	1980±4c	189± 5c	0.0±0.0
3rd	C	13	175±7a	1047±8a	2010±4a	4805±5a	697±11a	0.0±0.0
	LD_{10}	11	140±4b	840±5b	1610±3b	3805±5b	560±5b	0.0±0.0
	LD_{30}	10	94±5c	566±6c	1085±3c	2593±7c	377±4c	0.0±0.0
5th	C	13	86±4a	436±5a	698±3a	2969±6a	3581±3a	0.0±0.0
	LD_{10}	11	70±3b	350±4b	560±6b	2660±4b	3339±3b	0.0±0.0
	LD_{30}	10	47±4c	236±3c	377±5c	1792±3c	2263±4c	0.0±0.0

[a]TS: treatment stages; T: treatments; n: number of males used; C: control; LD_{10}: 1.00 ng larva^{-1}; LD_{30}: 3.75 ng larva^{-1}; Sg: spermatogonia; PS: primary spermatocytes; SS: secondary spermatocytes; St: spermatids; ECMS: elongated cysts with mature sperm; ESB: eupyrene sperm bundles; pupation occurred usually during 0200 to 0800 hour and collected between 0800 to 1000 hour.
[b]Data were analyzed using one-way ANOVA (Concepts, 1989) at P<0.0001. Means within columns followed by different letters are significantly different by Scheffe`s F-test (Scheffe, 1953) at 5%.

Table 8. Effect of sublethal doses of chlorfluazuron on spermatogenesis in the testes of pupae during 1st, 5th and 10th days of development after topical application to newly ecdysed fifth-instar larvae of *Spodoptera litura* (Source: Perveen, 2000b).

TS[a]	T[a]	n[a]	Sg[b] (M±SD)	PS[b] (M±SD)	SS[b] (M±SD)	St (M±SD)	ECMS (M±SD)	ESB (M± SD)
0	C	13	0±0a	174±4a	524±3a	1048±4a	2097±5a	4893±3a
	LD_{10}	11	35±3b	140±7b	385±3b	840±3b	1680±6b	3920±4b
	LD_{30}	10	24±2c	94±3b	259±2c	566±6c	1132±3c	2641±2c
1st	C	13	0±0a	0±0a	174±6a	611±3a	1047±6a	6902±5a
	LD_{10}	11	14±3b	28±3b	98±1b	490±5b	840±3b	5530±4b
	LD_{30}	10	9±2c	19±2c	66±2c	330±4c	566±2c	3728±5c
2nd	C	13	0.0±0.0	0.0±0.0	43±3a	219±4a	437±6a	8037±6a
	LD_{10}	11	0.0±0.0	0.0±0.0	35±3b	175±3b	350±5b	6440±5b
	LD_{30}	10	0.0±0.0	0.0±0.0	24±2c	118±4c	236±4c	4338±4c

[a]TS: treatment stages; T: treatments; n: number of males used; C: control; LD_{10}: 1.00 ng larva^{-1}; LD_{30}: 3.75 ng larva^{-1}; M: mean; Sg.: spermatogonia; PS: primary spermatocytes; SS: secondary spermatocytes; St.: spermatids; ECMS: elongated cysts with mature sperm; ESB: eupyrene sperm bundles; adults emerged usually between 2300 to 0200 hour and and between collected 0800 to 1000 hour.
[b]Data were analyzed using 1-way ANOVA (Concepts, 1989) at P<0.0001. Means within columns followed by different letters are significantly different by Scheffe`s F-test (Scheffe, 1953) at 5%.

Table 9. Effect of sublethal doses of chlorfluazuron on spermatogenesis in the testes of adults during 0, 1st and 2nd day of development after topical application to newly ecdysed fifth-instar larvae of *Spodoptera litura* (Source: Perveen, 2000b).

In the testis of newly emerged LD_{10}- treated adults, the number of eupyrene and apyrene sperm bundles was significantly reduced (P<0.001), and even more significantly reduced

($P<0.0001$) in LD_{30}- treated adults compared with the controls. When males were treated with the LD_{10} or LD_{30}, the ratio of eupyrene to apyrene sperm bundles was not significantly changed; apyrene sperm bundle comprised about half of the total sperm complement (Table 10).

T[a]	n[a]	NESB[b] (M±SD)	NASB[b] (M±SD)	Ratios (%)= ESB:ASB
Control	30	4893±546a	4697±520a	51.1:48.9
LD_{10}	30	3920±426b	3763±466b	51.0:48.9
LD_{30}	30	2641±161c	2386±271c	52.5:47.5

[a]LD_{10}: 1.00 ng larva^{-1}; LD_{30}: 3.75 ng larva^{-1}; T: treatments; n: number of males used; NESB: number of eupyrene sperm bundle; NASB: number of apyrene sperm bundle; ESB: eupyrene sperm bundle; ASB: apyrene sperm bundle

[b]Data were analyzed using one-way ANOVA (Concepts, 1989) at $P<0.0001$. Means within columns followed by different letters are significantly different by Scheffe`s F-test (Scheffe, 1953) at 5%.

Table 10. Effect of sublethal doses of chlorfluazuron and comparison of the number of eupyrene and apyrene sperm bundles in the testis of newly emerged unmated adults after topical application to newly ecdysed fifth-instar larvae of *Spodoptera litura* (Source: Perveen, 2000b).

Developmental stages of sperm	T[a]	n[a]	Size (dm in μm)	
			(M±SD)[b]	ranges (min–max)
Spermatogonia (S)	Control	10	7.1±0.7a	6–8
	LD_{10}	10	5.9±0.8b	5–7
	LD_{30}	10	4.1±0.9c	3–5
Primary spermatocytes (D)	Control	10	15.1±0.7a	14–16
	LD_{10}	10	13.0±1.3b	11–14
	LD_{30}	10	11.4±1.5c	10–48
Secondry spermatocyte (B_2)	Control	10	31.0±1.8a	28–33
	LD_{10}	10	29.2±1.6b	27–31
	LD_{30}	10	25.0±1.2c	24–27
Spermatids (P_{10})	Control	10	4.1±0.9a	3–5
	LD_{10}	10	2.9±0.87b	2–4
	LD_{30}	10	1.9±0.7c	1–3

[a]LD_{10}: 1.00 ng larva^{-1}; LD_{30}: 3.75 ng larva^{-1}; T: treatments; n: number of males used; for S, D and B_2 refer to Table 5.1; P_{10}: ten day old pupae

[b]Data were analyzed using one-way ANOVA (Concepts, 1989) at $P<0.0001$. Means within columns followed by different letters are significantly different by Scheffe`s *F*-test (Scheffe, 1953) at 5%.

Table 11. Effect of sublethal doses of chlorfluazuron on the size of various developmental stages of sperm observed in the testes of sixth-instar larvae and puape after topical application to newly ecdysed fifth-instar larvae of *Spodoptera litura* (Source: Perveen, 2000b).

The size of the spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids was significantly reduced ($P<0.001$) in LD_{10}-treated and more significantly reduced ($P<0.0001$) in LD_{30}-treated insects compared with the controls (Table 11) in newly emerged adults (Perveen, 2000b).

In newly emerged adults, the width and length of elongated cysts with mature sperm, eupyrene and apyrene sperm bundles were significantly reduced ($P<0.001$) in LD_{10}- and more significantly reduced ($P<0.0001$) in LD_{30}- treated insects compared with the controls (Table 12) (Perveen, 2000b).

Developmental stages of sperm	T[a]	n[a]	sizes of various developmental stages			
			length (μm)[b]		width (μm)[b]	
			M±SD	Ranges (min–max)	M±SD	Ranges (min–max)
Elongated spermatocytes	Control	10	45.5±2.4a	(42–50)	40.0±2.0a	(35–42)
	LD_{10}	10	42.2±1.7b	(40–44)	37.2±1.3b	(35–39)
	LD_{30}	10	40.0±0.8c	(39–41)	35.2±1.6c	(34–38)
Eupyrene sperm bundles	Control	10	98.0±4.4a	(92–105)	33.2±1.9a	(30–36)
	LD_{10}	10	94.7±1.4b	(93–97)	31.3±1.4b	(29–33)
	LD_{30}	10	91.5±1.0c	(90–93)	29.1±1.2c	(28–31)
Apyrene sperm bundles	Control	10	24.9±1.2a	(23–27)	16.1±1.7a	(14–19)
	LD_{10}	10	94.7±1.4b	(21–25)	14.2±1.3b	(12–16)
	LD_{30}	10	20.3±0.9c	(19–22)	12.2±1.2c	(11–14)

[a]LD_{10}: 1.00 ng larva^{-1}; LD_{30}: 3.75 ng larva^{-1}; T: treatments; n: number of males used
[b]Data were analyzed using one-way ANOVA (Concepts, 1989) at $P<0.0001$. Means within columns followed by different letters are significantly different by Scheffe`s *F*-test (Scheffe, 1953) at 5%.

Table 12. Effect of sublethal doses of chlorfluazuron on size of various developmental stages of sperm observed in the testes of newly emerged adults after topical application to newly ecdysed fifth-instar larvae of *Spodoptera litura* (Source: Perveen, 2000b).

T[a]	n[a]	Eupyrene sperm bundles in vas deferens		
		Pre-adult[b] (M±SD)	Newly emerged adult[b] (M±SD)	One day old adult[b] (M±SD)
Control	30	102±29a	1002±116a	2513±407a
LD_{10}	30	0b	23±4.9b	1621±159b
LD_{30}	30	0b	0c	1080±75c

[a]LD_{10}: 1.00 ng larva^{-1}; LD_{30}: 3.75 ng larva^{-1}; T: treatments; n: number of males used
[b]Data were analyzed using one-way ANOVA (Concepts, 1989) at $P<0.0001$. Means within columns followed by different letters are significantly different by Scheffe`s *F*-test (Scheffe, 1953) at 5%.

Table 13. Effect of sublethal doses of chlorfluazuron on the number of eupyrene sperm bundles in the vas deferens during different developmental days of adults after topical application to newly ecdysed fifth-instar larvae of *Spodoptera litura* (Source: Perveen, 2000b)

In the vas deferens of male pre-adult controls, the mean number of eupyrene sperm bundles was 102±29, but no sperm bundles observed in the LD_{10}- or LD_{30}-treated males of the same age (Table 13) (Perveen, 2000b).
In newly emerged control males, the mean number of eupyrene sperm bundles was 1002±116, and in LD_{10}-treated adult males, 23 ± 4.9. In LD_{30}-treated adults male there was no sperm bundles were observed. Moreover, in 1 day-old LD_{10}-treated adult males, the number of eupyrene sperm bundles was significantly ($P<0.001$) reduced and more significantly ($P<0.0001$) reduced in LD_{30}-treated males compared with the controls (Table 14) (Perveen, 2000b).
In the testis and vas deferens of newly emerged, LD_{10}-treated males, the total number of eupyrene sperm bundles was significantly reduced ($P<0.001$) and more significantly reduced ($P<0.0001$) in LD_{30}-treated males had no sperm bundles in the vas deferens compared with the controls (Table 14) (Perveen, 2000b).

T[a]	n[a]	NESB in testis[a,b] (M±SD)	NASB in vasa deferens[a,b] (M±SD)	TNESB (testis+ vasa deferens)[a,b] (M±SD)
Control	30	4893±546a	1002±116a	5791±640a
LD_{10}	30	3920±426b	23±4.9b	3943±425b
LD_{30}	30	2641±161c	0±0c	2641±161c

[a]LD_{10}: 1.00 ng larva^{-1}; LD_{30}: 3.75 ng larva^{-1}; T: treatments; n: number of males used; NESB: number of eupyrene sperm bundle; NASB: number of apyrene sperm bundle; ESB: eupyrene sperm bundle; ASB: apyrene sperm bundle; TNESB: total number of eupyrene sperm bundle
[b]Data were analyzed using one-way ANOVA (Concepts, 1989) at $P<0.0001$. Means within columns followed by different letters are significantly different by Scheffe`s *F*-test (Scheffe, 1953) at 5%.

Table 14. Effect of sublethal doses of chlorfluazuron on the total number of eupyrene sperm bundles in the testis and vas deferens of newly emerged adults after topical application to newly ecdysed fifth-instar larvae of *Spodoptera litura* (Source: Perveen, 2000b)

4.3 Discussion

Topical application of sublethal doses of chlorfuazuron (LD_{10}: 1.00 ng larva^{-1}; LD_{30}: 3.75 ng larva^{-1}) has an effect on reproduction of *S. litura* by reducing the fecundity, fertilityand hatchability. Fecundity was reduced to a similar degree (35±44%) when females, males or both sexes were treated with LD_{10} or LD_{30}. Fertility was reduced by 42% or 52% when females were treated with LD_{10} or LD_{30}, respectively, and by 60% or 63%, respectively, when males or both sexes were treated with LD_{10}. Fertility was reduced by 78% or 80% when males or both sexes were treated with LD_{30}. The hatchability was reduced by 20% or 23% when females were treated with LD_{10} or LD_{30}, respectively, and by 37% or 39%, respectively, when males or both sexes were treated with LD_{30}. Hatchability was reduced by 55% or 56% when males or both sexes were treated with LD_{30} (Perveen, 2000a). Thus, this study was conducted to determine the causes of the reduction in these reproductive parameters. Topical application of sublethal doses of chlorfuazuron has an effect on testis development by decreasing the volume and weight of testes and its sheath thickness, and also on spermatogenesis by decreasing the number of cysts, eupyrene and apyrene bundles during different times in development. Reduction in the testes volume and weight might be caused

by a reduction in the number of cysts, number and size of sperm bundles, thickness of testes sheath, and/or the reduction in protein content of testes constituents. When females are treated with LD_{10} of chlorfuazuron, no signifiant reduction in the inseminated eupyrene sperm number is observed compared with controls. However, LD_{10} and LD_{30} treatment of males significantly reduces (65.8% and 88.6%) the number of inseminated eupyrene sperm. Moreover, no significant ($P<0.0001$) differences in the reduction are observed on the inseminated eupyrene sperm number when males or both sexes are treated either with LD_{10} or LD_{30} (Perveen, 2008). Therefore, the main cause of the reduction in the fecundity, fertility and hatchability is a decrease in inseminated eupyrene sperm numbers. In larval *S. litura*, the bright yellow-coloured testes are distinctly paired, reniform and situated between the 5th and 6th abdominal segments. Each of the lateral testicular lobes is made up of four follicles. The testicular lobes are enclosed within two thick, double-layered peritoneal sheaths. Each of these sheaths comprises two layers of epithelial cells. The external sheath is made up of lightly stained cuboidal cells resting on a basement membrane. This forms a common envelope to all the follicles of the testis and is penetrated by tracheal branches. The inner sheath is made up of more darkly stained elliptical cells, within which muscle fibres are distinguisable. Ingrowths from this sheath in the form of double-walled septa penetrate between the follicles to separate them from one another. The testes sheath reaches its maximum thickness at day 0 of pupation in the controls, whereas in LD_{10}- treated males maximum thickness occurs later at 1 day. In LD_{30}-treated males maximum thickness occurs at 2 day after pupation. Difubenzuron affects ecdysteroid secretion from the epidermis in Tenebrio molitor (Soltani, 1984), ovaries of *Cydia pomonella* (Soltani et al., 1987; Soltani et al., 1989b) and also the concentrations of haemolymph constituents in *T. molitor* (Soltani, 1992). Ecdysteroids have been reported to stimulate spermatogenesis in many insect species (Dumser, 1980b; Gelman and Hayes, 1982). In Mamestra brassica (Shimizu et al., 1985), *Heliothis virescens* (Loeb, 1986) and *L. dispar* (Loeb et al., 1988) testes synthesize ecdysteroids. The treatment with chlorfuazuron effects ecdysteroid production by the testis sheet remains to be investigated. Gelman and Hayes (1982) and Gelman et al. (1988) observed in *Ostrinia nubilalis*, the size and weight of separate testes paired. Topical application of the sublethal dose, LD_{10} of chlorfuazuron significantly reduces ($P<0.0001$) the weight and size of testes, and this is even greater in LD_{30}-treated males. However, the topical application of similar sublethal doses of chlorfuazuron significantly reduced ($P<0.0001$) ovarian weight in post-pupal and developing pharate adult females as compared with that of the controls (Perveen and Miyata, 2000). However, significant differences are not observed in ovarian weight when adult females are treated with LD_{10} or LD_{30} doses. The maturation of insect testes 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 newly emerged males from LD_{10}- or LD_{30}-treated larvae the eupyrene and apyrene sperm bundles are significantly ($P<0.0001$) smaller in size and number compared with those of controls. Spermatozoa descend regularly from the testis through the vas deferens into the seminal vesicles, which fill with eupyrene sperm bundles with cysts and individual apyrene sperm. Yoshida (1994) also reported that the number of eupyrene sperm bundles in the testis and vas deferens of newly emerged treated (LD_{10}) males is reduced by 36%, and the initiation of sperm movement from testis to seminal vesicle was delayed. The present results show that initiation of sperm movement from testis to vas deferens is delayed after chlorofuazuron treatment and this is

caused by the delay of spermatogenesis. When males or both sexes are treated on the first day of pairing, few or none mate. However, seven to nine pairs mate in control or female treated crosses. During the next day, seven to 10 pairs mate in 10±13 pairs of seven combinations of crosses (Perveen, 2008). These results suggest that the delay of the first mating is caused by the delayed spermatogenesis. The effect of chlorfuazuron on testicular development and spermatogenesis is one of the factors responsible for the reduction in fecundity, fertility and hatchability caused by the sublethal doses of chlorfuazuron. More work is in progress to determine the biochemical mechanism of these effects in *S. litura*.

4.4 Conclusion

The physiological mechanism of action of chlorfluazuron describes here on testicular development and spermatogenesis when sublethal doses (LD_{10}: 1.00 ng larva^{-1} or LD_{30}: 3.75 ng larva^{-1}) are applied topically to the cuticle of newly ecdysed fifth instars of *Spodoptera litura*. These doses disrupt the growth and development of testes by decreasing the volume and weight of testes and thickness of testes sheath as compared with that of the controls. Additionally, such doses disrupt spermatogenesis by reducing the number and size of eupyrene and apyrene sperm bundles in the testis. Very few or no eupyrene sperm bundles are observed in vas deferens of pre- and newly ecdysed adults compared with controls. This result shows that the transfer of sperm bundles from testes to vas deferens is delayed in treated males. The effects of chlorfluazuron on testicular development and spermatogenesis are thought to be one of the factors responsible for the reduction in fecundity, fertility and hatchability caused by sublethal doses of chlorfluazuron.

5. References

Abro, G. H., Memon, A. J. and Syed, T. S. (1997). Sublethal effects of cyhalothrin and fluvalinate on biology of *Spodoptera litura* F. Pakistan J. Zool. 29: 181–184.

Anderson, V. L. and McLean, R. L. (1974). Design of experiments: a realistic approach. Dekker, New York. p. 70.

Amaldoss, G. (1989). Ultrastructure and physiology of the ductus ejaculatorius duplex and seminal vesicle of male *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae). Proc. Indian Acad. Sci. (Anim. Sci.) 98 (6): 405–418.

Anonymous, (1969). Summary of panel meeting in "Sterile male technique for eradication or control of harmful insect". Intl. Atom. Ener. Com. Vienna. pp. 80

Baloch, S. H. and Abbasi, Q. D. (1977). Investigation of the ecology and biology of the cutworm (Noctuidae: Lepidoptera) in the Hyderabad region. First Annu. Res. Report, Pakistan Sci. Found. Islamabad. p. 80.

Barbosa, P. (1974). Dissecting fluids and saline solutions. *In*: "Manual of basic techniques in insect' histology". (Mass, A. D. ed.), Oxford Publisher, London. p. 243.

Bargers, A. and Raw, F. (1967). Soil biology. Academic Press. New York. pp. 53. Beeman, R. W. (1982). Advances in mode of action of insecticides. Ann. Rev. Entomol. 27: 253–281.

Benz, G. (1969). Influence of mating, insemination and other factors on oÖgenesis and oviposition in the moth *Zeiraphera diniana*. J. Ins. Physiol. 15: 55–71.

Binnington, K. and Retnakaran, A. (1991). Epidermis: A biologically Active target for metabolic inhibitors. *In*: "Physiology of the insect epidermis" (Binnington, K. and Retnakaran, A. eds.) CSIRO Publications, Australia. p. 307–334.

Brushwein, J. R. and Granett, J. (1977). Laboratory tests of two moulting disruptants, El-494 and dimilin, against the spruce budworm (Lepidoptera: Tortricidae): mortality and reproductive effects. Me. Life Sci. Agr. Exp. St. Mis. Report. p. 192.

Chase, J. A. and Gilliland, F. R. J. (1972). Testicular development in Thet tobacco budworm. Ann. Entomol. Soc. Amer. 65: 901– 906.

Chatani, F. and Ohnishi, E. (1976). Effects of ecdysone on theovarian development of *Bombyx* silkworm. Develop. Gro. Diff. 18: 48–484.

Chaudhury, M. F. B. and Raun, A. S. (1966). Spermatogenesis And testicular development of the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyraustidae). Ann. Entomol. Soc. Amer. 59: 1157–1159.

Clark, L., Temple, G. H. R. and Vincent, J. F. V. (1977). The effects of a chitin inhibitor, dimilin on the reproduction of the peritrophic membrane in the locust, *Locusta migratoria*. J. Insect Physiol. 24: 241–246.

Concepts, A. (1989). Super ANOVA. Abacus Concepts, Berkeley, *CA*. pp. 120.

Crystal, M. M. (1978). Diflubenzuron-induced decrease of egg hatch of screw worms (Diptera: Calliphoridae). J. Med. Entomol. 15: 52–56.

Deb, D. C. and Chakaravorty, S. (1981). Effects of a juvenoid on the growth and differentiation of the ovary of *Corcyra cephalonica* (Lepidoptera). J. Insect Physiol. 27: 103–111.

Doncaster, L. (1911). Some stages in the spermatogenesis of Abraxas *grossulariata* and its variety *lacticolor*. J. Gent. 1: 179–184.

Dumser, J. B. (1980 a). The regulation of spermatogenesis in insects. Ann. Rev. Entomol. 25: 341–369.

Dumser, J. B. (1980 b). *In vitro* effects of ecdysterone on the spermatogonial cell in *Locusta*. Intl. J. Invert. Rep. 2: 165–174.

Emam, A. K., El-Refal, S. A. and Degheele, D. (1988). Effect of Sublethal dosages of four chitin synthesis inhibitors on the reproduction potential and F-1 generation of the Egyptian cotton leafworm *Spodoptera litoralis* (boisd.) (Lepidoptera: Noctuidae). Med. Van de Facland. Rij. Gent. 53: 249–254.

Engelman, F. (1979). Insect vitellogenin: identification, bio-synthesis, and role in vitellogenesis. Adv. Insect Physiol. 27: 49–108.

Etman, A. A. M. and Hooper, G. H. S. (1979). Developmental and reproductive biology of *Spodoptera litura* (F.) (Lepidoptera: Noctuidae). J. Aus. Entomol. Soc. 18: 363–372.

Friedlander, M. and Gitay, H. (1972). The fate of the normal-a-nucleated spermatozoa in inseminated females of the silkworm, Bombyx mori. J. Morphol. 138: 121–129.

Fytizas, E. (1976). L'action du TH6040 sur la metamorphose de *Dacus oleae* Gemel. (Diptera, Trypetidae). *Z. Angew. Entomol. 31*: 440–444. (Abstract in English read).

Gage, M. J. G. and Cook, P. A. (1994). Sperm size or number? Effects of nutritional stress upon eupyrene and apyrene sperm production strategies in the moth, *Plodia interpunctella* (Lepidoptera: Pyralidae). Func. Ecol. 8: 594–599.

Ganiev, S. G. (1986). Residual amount of dimilin in the plantand soil of paistaccio stands in SW Kirgizia. *Lesn. Khoz. 10*: 63–70.

Gelman, D. B. and Hayes, D. K. (1982). Methods and markers for synchronizing the maturation of fifth-stage diapause-bound and non diapause-bound larvae, pupae and pharate adults of the European cornborer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae). Ann. Entomol. Soc. Amer. 75: 485–493.

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.

Goldschmidt, R. (1916). The function of the apyrene spermatozoa. Science 44: 544–546.

Gupta, H. C., Bareth, S. S. and Sharma, S. K. (1991). Bioefficacy of edible and non-edible oils against pulse, *Callosobrochus chinensis* L. on stored pulses and their effect on germination. Agri. Biol. Res. 7: 101–107.

Haga, T., Toki, T., Tsujii, Y. and Nishiyama, R. (1992). Development of an insect growth regulator, chlorfluazuron. J. Pestic. Sci.17: S103–S113.

Hajjar, N. P. and Casida, J. E. (1979). Structure-activity relationships of benzoyl phenyl urea as toxicants and chitin synthesis inhibitors in *Oncopeltus fasciatus*. Pestic. Biochem. Physiol. 11: 33–45.

Hashizume, B. (1988). Atabron® 5E, a new IGR insecticide (chlorfluazuron). Jpn. Pestic. Inform. 58: 32–34.

Hatakoshi, M. (1992). An inhibitory mechanism over oviposition in the tobacco cutworm, *Spodoptera litura* by juvenile hormone analogue pyriproxyfen. J. Insect. Physiol. 38: 793–801.

Hatakoshi, M. and Hirano, M. (1990). Effects of S-71639 on the reproduction of *Spodoptera litura.* Adv. Invert. Rep. 5: 429–434.

Haines, L. C. (1981). Changes in colour of a secretion in the reproductive tract of adult males of*Spodoptera littoralis* (Boisduval) (Lepidoptera: Notuidae) with age and mated status. Bull. Entomol. Res. 71: 501–598.

Haynes, K. F. (1988). Sublethal effect of neurotoxic insecticides on insect behaviour. *Ann. Rev. Entomol. 33*: 149–168.

He, Y. (1994). Reproductive strategies in the armyworm, *Pseudaletia separata* (Lepidoptera: Noctuidae): with special reference to rearing density. Ph. D. Thesis. Graduate School of Agricultural Sciences, Nagoya University, Japan. pp. 122.

He, Y., Tanaka, T. and Miyata, T. (1995). Eupyrene and apyrene sperm and their numerical fluctuations inside the female reproductive tract of the armyworm, *Pseudaletia separata.* J. Insect. Physiol. 41: 689–694.

Holst, H. Z. (1974). Die fertility ätsbeeinflussende Wirkung des neuen insektizids P D 60-40 bei *Epilachna varivestis* Muls (Col. occinellidae) und *Leptinotarsa decemlineata* Say. (Col. Chrysomelidae). Z. Pflkranzk. Pflschutz. 81: 1–7. (Abstract in English read)

Holst, H. Z. (1974). Die fertility ätsbeeinflussende Wirkung des neuen insektizids P D 60-40 bei *Epilachna varivestis* Muls (Col. occinellidae) und *Leptinotarsa decemlineata* Say. (Col. Chrysomelidae). Z. Pflkranzk. Pflschutz. 82: 1–7. (Abstract in English read)

Holt, G. G. and North, D. T. (1970a). Spermatogenesis in the cabbage looper, *Trichoplusia ni*. (Lepidoptera: Noctuidae). Ann. Entomol. Soc. Amer. 63: 501–507.

Holt, G. G. and North, D. T. (1970b). Effects of gamma Irradiation on the mechanisms of sperm transfer in *Trichoplusia ni*. J. Insect. Physiol. 16: 2211–2222.

Hunter, E. and Vincent, J. F. V. (1974). The effects of a novel insecticide on insect cuticle. Experientia 4: 484–490.

Indrasith, L. S., Sasaki, T., Yaginuma, T. and Yamashita, O. (1988). The occurrence of a premature form of egg-specific protein in vitellogenic follicles of *Bombyx mori*. J. Comp. Physiol. 158: 1–7.

Jakob, W. L. (1973). Development inhibition of mosquitoes and house fly by urea analogues. *J. Med. Entomol. 10*: 540–543.

James, E. W. (1974). Laboratory and field evaluation of TH-6040, against House fly and stable fly. J. Econ. Entomol. 67: 746–747.

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"* (Evans, P. D. and Wigglesworth, V.B. eds.), Academic Press, London. v. 22, p. 299–396.

Karlinsky, A. (1963). Effects de l'ablation des corpora allata imaginaux sur le developement ovarien de *Pieris brassicae* L. (Lepidoptera). *C. R.* Acad. Sci. Paris. *256*: 4101–1103. (Abstract in English read)

Kasuga, H., Osani, M., Yonezawa, Y. and Aigaki, T. (1985). An energy supply device for spermatozoa in the spermatophore of the silkworm. Develop. Gro. Diff. 27: 514–522.

Katsuno, S. (1977a). Studies on eupyrene and apyrene spermatozoa in the silkworm *Bombyx mori* L. (Lepidoptera: Bombycidae). I. The intra testicular behaviour of the spermatozoa at various stages from the 5th instar to the adult. Appl. Entomol. Zool. 12: 142–147.

Katsuno, S. (1977 b). Studies on eupyrene and apyrene spermatozoa in the silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae). II. The intra testicular behaviour of the spermatozoa after emergence. Appl. Entomol. Zool. 12: 148–153.

Katsuno, S. (1977c). Studies on eupyrene and apyrene spermatozoa in the silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae). III. The post testicular behaviour of spermatozoa at various stages from the pupa to adult. Appl. Entomol. Zool. 12: 142–247.

Katsuno, S. (1977d). Studies on eupyrene and apyrene spermatozoa in the silkworm *Bombyx mori* (Lepidoptera: Bombycidae). IV. The behaviour of the spermatozoa in the internal reproductive organs of female moths. Appl. Entomol. Zool. 12: 352–359.

Ker, F. (1977). Investigation of locust cuticle using the insecticide diflubenzuron. *J. Insect Physiol. 23*: 39–48.

Khan, M. F. and Naqvi, S. N. H. (1988). Toxicological studies of diflubenzuron and nicotine cyanurate on house flies larvae. Pakisrtan J. Entomol. 3: 95–106.

Khuhro, R. D., Abbasi, Q. D., Abro, G. H., Baloch, S. H. and Soomor, A. H. (1986). Population dynamics of *Spodoptera litura* (F.) as influenced by ecological conditions and its host plant preference at Tandojam. Pakisrtan J. Entomol. 18: 351–357.

Kunkel, J. G. and Nordin, J. H. (1985). Yolk proteins. *In: "Comprehensive Insect Biochemitry, Physiology and Pharmacology"* (Kerkut, G. A. and Gilbert, L. I. eds.), Pergamon Press, Oxford. v. 1, p. 83–111.

Leuschner, K. (1974). Effects of the new insecticide PDD 60-40 on nymphs and adults of the cabbage bug, *Eurydema oleraceum* L. (Heteroptera, Pentatomidae). Z. Pflkrankh. Pflschutz. 81: 8–12.

Leviatan, R. and Friedlander, M. (1979). The eupyrene-apyrene dichotomous spermatogenesis of Lepidoptera. I. The relationship with post embryonic development and the role of the decline in juvenile hormone titre towards pupation. Develop. Biol. 68: 515–525.

Lim, S. J. and Lee, S. S. (1982). Toxicity of diflubenzuron to the grass hopper, *Oxy japonica*; effect on reproduction. Entomol. Exp. Appl. 31: 154–158.

Loeb, M. J. (1986). Ecdysteroids in testis sheaths of *Heliothis Virescens* larvae: An immunological study. Arch. Insect Biochem. Physiol. 3: 173–180.

Loeb, M., Brand, E. P. and Birnbaum, M. J. (1984). Ecdysteroid production by testes of budworm, *Heliothis virescens*, from last-larval instar to adult. *J. Insect Physiol. 30: 375–381.*

Loeb, M. J., Brandt, E. P., Woods, C. W. and Bell, R. A. (1988). Secretion of ecdysteroids by sheaths of testis of the gypsy moth, *Lymantria dispar*, and its regulation by testis ecdysiotropin. J. Exp. Zool. 248: 94–100.

Machida, J. (1929). Feine experimentelle untersuchung über die apyrene spermatozoen des seidenspinners *Bombyx mori* L. Zur. Beitru. Wissen. 9: 466–510. (Abstract in English read)

Madore, C. D., Drion, G. B. and John, B. D. (1983). Reduction of reproductive potential in spruce budworm (Lepidoptera: Tortricidae) by a chitin-inhibiting insect growth regulator. J. Econ. Entomol. 76: 708–710.

Matsuura, H. and Naito, A. (1997). Studies on the cold-hardiness and overwintering of *Spodoptera litura* F. (Lepidoptera: Noctuidae) VI. possible overwintering areas predicted from meteorological data in Japan. Appl. Entomol. Zool. 32: 167–177.

Matthee, J. J. (1951). The structure and physiology of the egg of *Locustana pardalina* (Walk.). Bull. Develop. Agri. For. Un. S. Afr. no 316: p. 83.

Mellanby, K. (1976). Pesticides, the environment and the balance of nature.*In: "Pesticides and Human Welfare"* (Gunn, D. I. and Steven, J. G. R. eds) Oxford University Press, p. 217–227.

Meves, F. (1902). Über oligpyrene und apyrene spermien und über ihre entstehung, nach beobachtugen an *Paludina* und *Pygaera*. *Arch. Mikro. Anatom. 61*: 1–84. (Abstract in English read)

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.

Munson, J. P. (1906). Spermatogenesis of the butterfly, *Papilio rutulus*. Proc. Boston Soc. Natu. His. 33: 43–124.

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.

Nickle, D. A. (1979). Insect growth regulators: New protectants against the almond moth in stored inshell peanuts. J. Econ. Entomol. 72: 816–819.

Nijahout, M. M. and Riddiford, L. M. (1974). The control of egg maturation by juvenile hormone in the tobacco hornworm, *Manduca sexta*. Biol. Bull. 146: 377–392.

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.

Nomura, M. (1994). Physiological effects of pyriproxyfen against the common cutworm, *Spodoptera litura. M. Sc*. Thesis, Laboratory of Applied Entomology, Nagoya University. pp. 125.

Olszak, R. W., Pawlik, B. and Zajac, R. Z. Z. (1994). The influence of some insect growth regulators on mortality and fecundity of the aphidophagous coccinellids, *Adalia bipunctata* L. and *Coccinella septempunctata* L. (Col., Coccinellidae). J. Appl. Entomol. 117: 58–63.

Ottens, R. J. and Todd, J. W. (1979). Effect of diflubenzuron on reproduction and development of *Graphognathus peregrinus* and *G. leucoloma*. J. Econ. Entomol. 72: 743–746.

Pan, M. L. (1977). Juvenile hormone and vitellogenin synthesis in the *Cecropia* silk moth. Biol. Bull. 153: 336–345.

Perveen, F. (2011) Effects of Sublethal Doses of Chlorfluazuron on Ovarioles in the Common Cutworm, *Spodoptera litura* (F.) (Lepidoptera: Noctuidae). Journal of Life Science, USA (Accepted).

Perveen, F. (2000a). Sublethal effects of chlorfluazuron on reproductivity and viability of *Spodoptera litura* (F.) (Lep., Noctuidae). J. Appl. Entomol. 124: 223–231.

Perveen, F. (2000b). Effects of sublethal dose of chlorfluazuron on testicular development and spermatogesis in the common cutworm, *Spodoptera litura*. J. Physiol. Entomol. 25: 315–323.

Perveen, F. (2005). Effects of chlorfluazuron on the growth of common cutworm, *Spodoptera litura* (Lepidoptera: Noctuidae) with special reference to the morphology and histology of the reproductive system. Ph.D. Thesis, Department of Zoology, University of Karachi.

Perveen, F. (2008). Effects of sublethal doses of chlorfluazuron on the number of inseminated sperm in *Spodoptera litura* (Lepidoptera: Nocutidae). J. Entomol. Sci. 11: 111–121.

Perveen, F. and Miyata, T. (2000). Effects of sublethal dose of chlorfluazuron on ovarian development and oögenesis in the common cutworm, *Spodoptera litura* (F.) Lepidoptera: Noctuidae). Ann. Entomol. Soc. Ame. 93: 1131–1137.

Qureshi, R. A., Qadri, S., Anwarullah, M. and Naqvi, S. N. H. (1983). Effect of neopesticides (JHA) on the morphology, emergence and fertility of *Musca domestica* L. (PCSIR strain) and its relation to phosphatases. Zool. Ang. Entomol. 95: 304–309.

Riakhel, A. S. and Dhadialla, T. S. (1992). Accumulation of yolk proteins in insect oÖcytes. Ann. Rev. Entomol. 37: 217–251.

Ross, D. C. and Brown, T. M. (1982). Inhibition of larval growth in *Spodoptera frugipera* by sublethal dietary concentrations of insecticides. J. Agr. Food Chem. 30: 193–196.

Scheffe, H. (1953). A method for judging all contrasts in the analysis of variance. Biometrika 40: 87–104.

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.

Silberglied, R. E., Shepherd, J. G. and Dickinson, J. L. (1984). Eunuchs: the role of apyrene sperm in Lepidoptera? Amer. Natur. 123: 255–265.

Skibbe, J. T., Challaghan, P. T., Eccles, C. D. and Laing, W. A. (1995).Visulization of pH in the larval midgut of *Spodoptera litura* using ^{31}P-NMR Microscopy. J. Insect Physiol. 42: 777–790.

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., Quennedey, A., Delbecque, J. P. and Delchambre, J. (1987). Diflubenzuron-induced alterations during *in vitro* development of *Tenebrio molitor* pupal integument. Arch. Insect Biochem. Physiol. 5: 201–209.

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. (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.

Sridevi, R., Gupta, A. D. and Ramamurty, P. S. (1989a). Spermatogenesis in *Spodoptera litura* (Lepidoptera: Noctuidae). Entomon 14: 01–09.

Sroka, P. and Gilbert, L. I. (1971). Studies on the endocrine control of post emergence ovarian maturation in *Manduca sexta*. J. Insect Physiol. 71: 2409–2419.

Taft, H. M. and Hopkins, R. A. (1975). Boll weevils: field populations controlled by sterilizing emerging over winter females with a TH-6040 sprayable bait. J. Econ. Entomol. 68: 551–554.

Tahir, S., Anwer, T. and Naqvi, S. N. H. (1992). Toxicity and Residual effects of novel pesticides against rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae). Pakistan J. Zool. 24: 111–114.

Takahashi, S. and Mizohata, H. (1975). Critical period necessary for the imaginal moulting and egg maturation of the eri-silkworm, *Samia cynthia* Ricini. Zool. Mag. 84: 283–295.

Telfer, W. H., Rubenstein, E. and Pan, M. L. (1981). How the ovary makes yolk in *Hylophora*. *In:* "*Regulation of Insect Development and Behaviour*" (Wroclaw, M. K. ed.) Technical University Press, p. 637–654.

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.

Wright, J. E. and Spates, G. E. (1976). Reproductive inhibition activity of the insect growth regulator TH-6040 against the stable and house fly: Effect on hatchability. J. Econ. Entomol. 69: 365–368.

Wright, J. E. and Retnakaran, A. (1987). "Chitin and Bezoyl phenyl Ureas" (Junk, K. eds.) The Hague. v. 1, pp. 225.

Yoshida, K. (1994). Studies on the physiological effects of chlorfluazuron: with special reference to reproductive effects of chlorfluazuron on *Spodoptera litura*. M. Sc. Thesis, Graduate School of Agricultural Sciences, Nagoya University. p. 69.

Younis, M. (1973). Losses of cotton crop due to insect pests. *Proc. Crop. Produc. Sem. Organ. ESSO (Pak.) Ltd*. p. 110.

Yu, S. J. (1983). Age variation in insecticide susceptibility and detoxification capacity of fall armyworm (Lepidoptera: Noctuidae) larvae. J. Econ. Entomol. 76:

3

Organophosphorus Insecticides and Glucose Homeostasis

Apurva Kumar R. Joshi and P.S. Rajini
Food Protectants and Infestation Control Department, Central Food Technological Research Institute (CSIR lab), Mysore, India

1. Introduction

The modern world has heavily thrived on the revolution in agricultural practices that have culminated in tremendous boost in agricultural productivity. Pesticides are perhaps one of the most important and effective strategies of the green revolution. Pesticides are the only class of toxic substances that are intentionally released into the environment for achieving greater good, a decision that far outweighs their toxicological concerns. Organophosphorus insecticides (OPI) are one of the most extensively used classes of insecticides. Chemically they are derivatives of phosphoric (H_3PO_4), phosphorous (H_3PO_3) or phosphinic acid (H_3PO_2) (Abou-Donia, 2003). The OPI were initially introduced as replacements for the much persistent organochlorine insecticides (Galloway & Handy, 2003). With systemic, contact and fumigant action, OPI find use as pest control agents in various situations. OPI are extensively used in agricultural practices for protecting food and commercial crops from various types of insects. In addition, OPI are also used in household situations for mitigating menacing pest varieties. They are not very stable chemically or biochemically and are degraded in soil, sediments and in surface water. Perhaps, it is this instability of these agents that has lead to their widespread and indiscriminate use, which has exposed animal and human life to various forms of health hazard. The increase in their use has lead to wide range of ecotoxicological problems and exposure to OPI is believed to be major cause of morbidity and mortality in many countries.

Huge scientific body of evidence suggests that OPI exposure is a major toxicological threat that may affect human and animal health because of their various toxicities such as neurotoxicity, endocrine toxicity, immunotoxicity, reproductive toxicity, genotoxicity and ability to induce organ damage, alterations in cellular oxidative balance and disrupt glucose homeostasis. Indeed, the data on residue levels of OPI in various sources reported from India does create a huge cause for concern regarding their toxic effects. Samples of raw and bottled water were reported to be contaminated with various OPI residues, some of which were much higher than recommended levels (Mathur et al., 2003). Sanghi et al. (2003) have reported OPI residue levels in breast milk samples in India. Based on the levels of OPI residues, it has been speculated that infants may consume 4.1 times higher levels of malathion than the average daily intake levels recommended by the World Health Organisation. Similarly, human blood samples were reported to be contaminated with residues of monocrotophos, chlorpyrifos, malathion and phosphamidon (Mathur et al.,

2005). Thus, OPI present a realistic environmental threat that could affect various facets of human health.

2. Toxicity of organophosphorus insecticides

The toxicity of active OPI is attributed to their ability to inhibit acetylcholinesterase (AChE, choline hydrolase, EC 3.1.1.7), an enzyme that catalyses the hydrolysis of the neurotransmitter acetylcholine (ACh), leading to cholinergic stress as a result of stimulation of muscarinic and nicotinic ACh receptors (Fukuto, 1990; Sogorb & Vilanova, 2003; Abou-Donia, 2003). The inhibition of AChE by an OPI takes place via a chemical reaction in which the serine hydroxyl moiety (of the active site) is phosphorylated. The phosphorylated enzyme is highly stable and, depending on the groups attached to the central 'P' atom of the OPI molecule, may be irreversibly inhibited.

There are several factors that determine the toxicity of OPI. Important of these are route and levels of exposure, structure of the substance and its interaction with the biotransformation/detoxification system of the body. The metabolic fate of OPI is basically the same in insects and animals. Following absorption, the distribution of OPI is variable. Blood half-lives are usually short, although plasma levels are in some cases maintained for several days. OPI undergo extensive biotransformation, which is complex and involves several metabolic systems in different organs, with simultaneous oxidative biotransformation at a number of points in the molecule, utilizing the cytochrome P-450 isoenzyme system. Metabolism occurs principally by oxidation, hydrolysis by esterases, and by transfer of portions of the molecule to glutathione. Oxidation of OPI may result in more or less toxic products. Most mammals have more efficient hydrolytic enzymes than insects and, therefore, are often more efficient in their detoxification processes. Numerous conjugation reactions follow the primary metabolic processes, and elimination of the phosphorus-containing residue may be via the urine or faeces. Some bound residues remain in exposed animals. Binding seems to be to proteins, principally, since there are limited data showing that incorporation of residues into DNA (Eto, 1974).

2.1 Neurotoxicity

Based on structure-function relationships, OPI are essentially neurotoxicants. Most important of their neurotoxicities is their 'cholinergic toxicity', which is a consequence of acetylcholinesterase (AChE) inhibition by OPI leading to accumulation of ACh and cholinergic stress. Signs of cholinergic toxicity include miosis, muscle fasciculation, excessive glandular secretions, nausea and vomiting (Namba, 1971). In addition, OPI are known to exert two other forms of neurotoxicities- Organophosphorus ester-induced delayed neurotoxicity (OPIDN) and Organophosphorus ester-induced chronic neurotoxicity (OPICN). OPIDN is a neurodegenerative disorder characterized by delayed onset of prolonged ataxia and upper motor neuron spasticity as a result of single or multiple exposures. OPICN refers to other forms of neurotoxicity that is distinct from both cholinergic toxicity and OPIDN. OPICN is characterized by neuronal degeneration and subsequent neurobehavioral and neuropsychological consequences (Abou-Donia, 2003).

2.2 Oxidative stress

Numerous studies provide evidence for the propensity of OPI to disrupt oxidative balance leading to oxidative stress (Soltaninejad & Abdollahi, 2009). Increased lipid peroxidation,

protein carbonylation, depletion of cellular antioxidant pools and alterations in enzymatic antioxidant status appear to be chief mechanisms of OPI-induced oxidative stress that often results in pathophysiological changes and organ damage. Several studies have demonstrated usefulness of antioxidant intervention in alleviating oxidative stress and pathophysiological changes induced by OPI (Kamath et al., 2008, Soltaninejad & Abdollahi, 2009). These studies lend unequivocal support to view that oxidative stress mediates as one of the chief mechanisms of OPI toxicity.

3. Organophosphorus insecticides and glucose homeostasis: mechanistic insights

In addition to neurotoxicity and oxidative stress, alterations in glucose homeostasis often culminating hyperglycemia is increasingly being reported as characteristic outcome of OPI toxicity. Meller et al., (1981) have described two cases of human subjects who were hospitalized with many complications including hyperglycemia. With no pseudocholinesterase detected, patients were given pralidoxime (AChE activator), which improved their condition and normalized hyperglycemia. Investigations revealed that they may have been exposed to malathion sprayed in their area. This case presents a classic case of hyperglycemic outcome following exposure to OPI as patients also exhibited miosis and muscle twitching. Numerous experiments have been conducted with experimental animals that reveal hyperglycemia as a characteristic outcome of OPI poisoning. A recent review by Rahimi & Abdollahi (2007) provides an exhaustive account of investigations revealing hyperglycemia in cases of OPI exposure.

There are certain characteristic features of alterations induced by OPI in glucose homeostasis. In cases of exposure to single dose of OPI, hyperglycemia appears to set in rapidly and peak changes are often followed by a trend of normalization. High dose of diazinon has been reported to cause hyperglycemia in mice that follows a trend of normalization (Seifert, 2001). Acute exposure of rats to malathion resulted in hyperglycemia with peak increase occurring at 2.2h after administration followed by decrease after 4h (Rodrigues et al., 1986). A similar case of reversible hyperglycemia has been reported by Lasram et al., (2008) following administration of a single dose of malathion to rats.

Biochemical changes associated with hyperglycemia serve as useful tools to understand etiology of OPI-induced hyperglycemia. Malathion has been reported to cause hyperglycemia in fasted rats. Interestingly, these hyperglycemic responses were not associated with hepatic glycogen depletion. The reversible phase of hyperglycemia was associated with increased glycogen deposition in liver, indicating that glucose may have come from gluconeogenesis (Gupta, 1974). Malathion induced hyperglycemia was associated with AChE inhibition in pancreas. More importantly, the trend of reversibility coincided with spontaneous reactivation of inhibited AChE (Lasram et al., 2008), indicating involvement of AChE-inhibition in hyperglycemia. Increase in blood glucose induced by sub chronic exposure of rats to malathion has been reported to be associated with increased glycogen phosphorylase and phosphoenolpyruvate carboxykinase activities, indicating involvement of both glycogenolytic and gluconeogenic processes. Increase in blood glucose levels induced by sub chronic exposure of rats to acephate has been reported to be associated with decrease in hepatic glycogen content (Deotare & Chakrabarti, 1981).

3.1 Pancreatic dysfunctions

Acute pancreatitis is also a well known complication of OP poisoning (Dressel et al., 1979; Frick et al., 1987; Hsiao et al., 1996), and epidemiological findings indicate that the incidence of pancreatitis is high in OPI intoxication based on various pathophysiological reports (Gokalp et al., 2005). The precise mechanisms underlying OPI-induced pancreatitis are still undefined, although it is believed to involve obstruction of pancreatic ducts and /or enhanced reactive oxygen species (Dressel et al., 1982; Sevillano et al., 2003, Sultatos, 1994). Involvement of oxidative stress following acute exposure to OPI has been reported recently (Banerjee et al., 2001) and it has been demonstrated unequivocally that lipid peroxidation is one of the molecular mechanisms involved in OPI-induced cytotoxicity (Akhgari et al., 2003; Ranjbar et al., 2002; Abdollahi et al., 2004b).

In view of the above, we attempted to understand the potential of repeated oral doses of dimethoate (DM) (at 20 and 40mg/kg b.w/d for 30days; doses corresponding to 1/20 and 1/10LD50 values) to cause alterations in glucose homeostasis and the associated biochemical alterations in pancreas of rats. We observed distinct signs of glucose intolerance among rats administered DM (**Fig. 1**) at time points at which un-treated rats showed normal glucose tolerance after an oral glucose load (3g/kg b.w.). We also observed that DM at both doses caused significant increase in blood glucose levels with concomitant inhibition of acetylcholinesterase activity and depletion of reduced glutathione contents in pancreas (**Table 1**) (Kamath & Rajini, 2007).

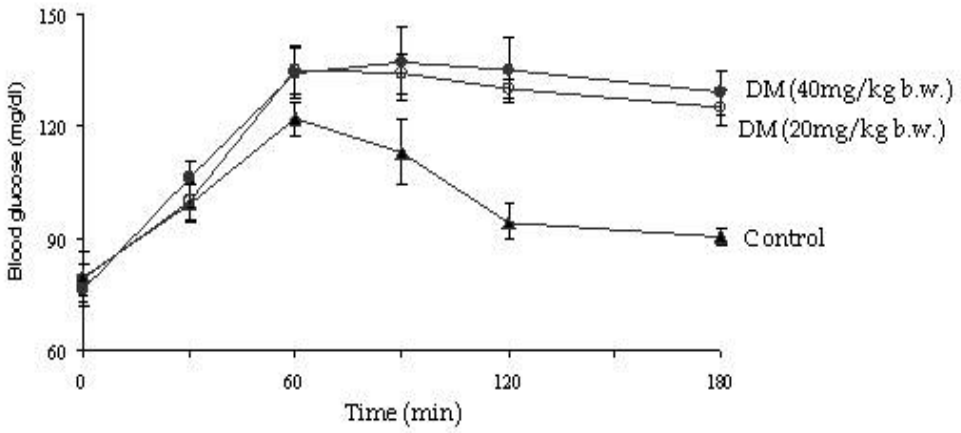

Fig. 1. Oral glucose tolerance at the end of 30 days in control (CTR) and Dimethoate (DM) treated rats.

Treatment	Blood glucose (mg/dl)		AChE (nmoles substrate hydrolyzed /min/mg protein)	GSH (mg/g tissue)
Dimethoate (mg/kg b.w.)	Initial	Final		
0	85.33 ± 3.85	91.33 ± 2.41	4.96 ± 1.47	1.11 ± 0.02
20	87.34 ± 5.23	105.28 ± 3.57[a]	2.94 ± 1.75	0.99 ± 0.05[a]
40	85.00 ± 5.30	138.67 ± 5.70[a,b]	0.43 ± 0.21[a,b]	0.91 ± 0.07[a,b]

Values are mean ± SEM (n=6);
[a] Comparison of control and other groups;
[b] Comparison of DM (20mg /kg b.w.) group with DM (40 mg/kg b.w.) group

Table 1. Blood glucose, acetylcholinesterase (AChE) and reduced glutathione (GSH) levels in pancreas of rats administered oral doses of Dimethoate (DM) for 30 days.

Further, DM also caused significant pancreatic damage as reflected by increased amylase (2-3 folds) and lipase (20 & 38%) activities in serum (**Fig 2**). These changes were sharply paralleled by significant damage in pancreatic milieu. There was a dose-related elevation in ROS levels in pancreas of treated rats. While the increase at the lower dose was 66%, a dramatic (150%) increase was evident at the higher dose. Concomitantly, a dose-related increase in TBARS (lipid peroxidation index) levels was observed in the pancreas of DM treated rats. There was 2.5 and 3.7 fold increase in TBARS level at lower and higher doses of DM respectively (**Fig. 3**). Activities of selected antioxidant enzymes were significantly elevated in the pancreas of treated rats compared to that of control rats. (**Table 2**) (Kamath & Rajini, 2007). These results are in accordance with the study of Hagar et al., (2002) who had earlier reported increased blood glucose levels and hyerinsulinemia with concomitant histochemical and ultramicrostructural changes in pancreas of rats following chronic exposure to dimethoate.

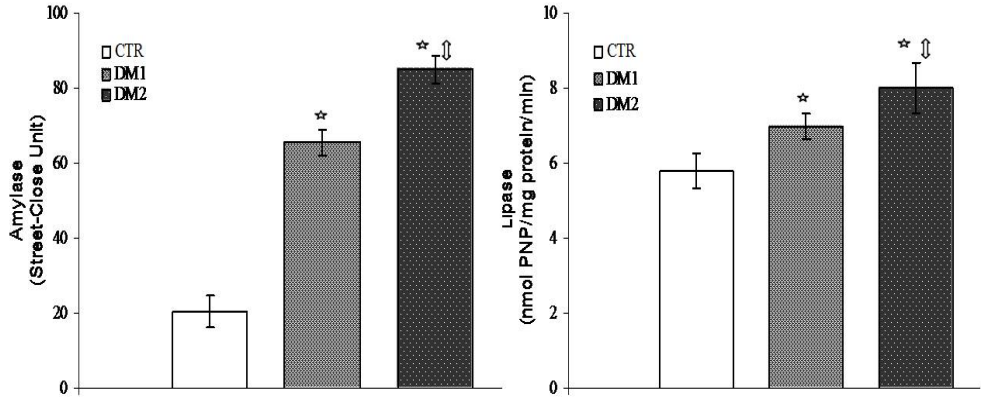

Fig. 2. Changes in pancreatic damage markers in rats induced by Dimethoate after 30 days (DM1: 20 mg/kg b.w/d; DM2: 40 mg/kg b.w/d). Values are mean ± SEM (n=6); * Comparison of control and other groups ($P < 0.01$), ⇳ Comparison of DM1 with DM2 ($P < 0.01$)

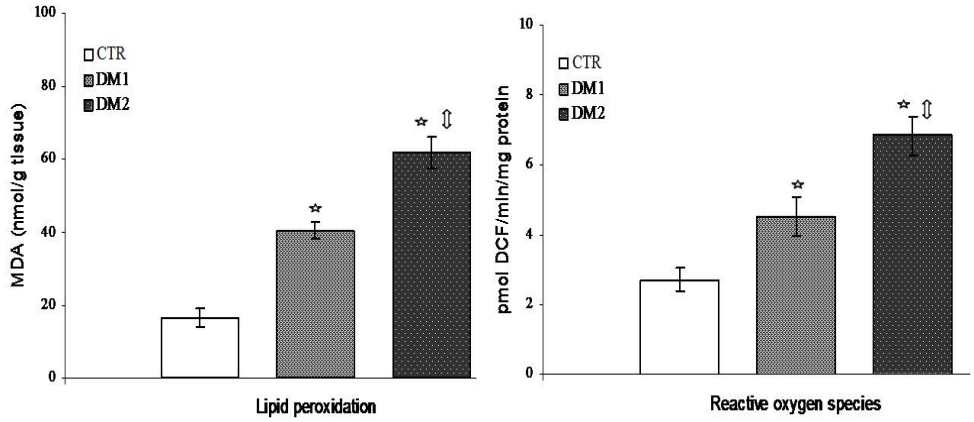

Fig. 3. Extent of lipid peroxidation and ROS levels in pancreas of control (CTR) and Dimethoate treated rats (DM1: 20 mg/kg b.w/d; DM2: 40 mg/kg b.w/d). Values are mean ± SEM (n=6); * Comparison of control and other groups ($P < 0.01$), ⇳ Comparison of DM1 with DM2 ($P < 0.01$).

Several studies have demonstrated pancreatitis after exposure to OPI (Dressel et al., 1979; Moore & James, 1988; Hsiao et al., 1996). Increase in the serum lipase and amylase activities reported by us clearly indicates that DM results in a state of pancreatic damage. Increased serum lipase activity has also been reported after administration of methidathion, an OPI (Mollaoglu et al., 2003). These results agree with earlier reports of acute pancreatitis in humans after accidental cutaneous exposure to DM (Marsh et al., 1988) and increase in amylase activity reported in dogs after diazinon administration (Dressel et al., 1982). Together, these studies clearly indicate that OPI possess propensity to elicit structural and functional alterations in pancreatic milieu that may be associated with disruptions in euglycemic conditions. From these studies, it may be argued that OPI may present a great threat to pancreatic functions in human beings and such threats may have far-reaching consequences on gluco-regulation in human beings.

Group	Enzyme Activity				
	SOD[1]	CAT[2]	GPX[3]	GR[3]	GST[4]
CTR	26.42 ± 2.2	9.38 ± 0.31	27.18 ± 5.24	17.50 ± 1.60	0.03 ± 0.004
DM1	42.72 ± 0.38[a]	10.24 ± 0.32	25.23 ± 3.89	19.72 ± 2.03	0.04 ± 0.003[a]
DM2	56.23 ±1.18[a,b]	15.44 ± 0.51[a,b]	13.85 ± 2.20[a,b]	25.30 ± 1.30[a,b]	0.06 ± 0.003[a,b]

[1]units/mg protein; [2]µmol/min/mg protein; [3]nmol/ min/ mg protein; [4]µmol/ min / mg protein
Values are mean ± SEM (n=6)
[a] Comparison of control (CTR) and other groups;
[b] Comparison of DM1 (DM: 20mg /kg b.w/d) group with DM2 (DM: 40 mg/kg b.w/d) group

Table 2. Antioxidant enzyme activities in pancreas of rats administered oral doses of Dimethoate for 30 days.

3.2 Adrenal involvement

Studies undertaken by several researchers to investigate the mechanisms mediating hyperglycemic effects of OPI have mainly focused on the involvement of cholinergic stress and adrenal functions. We have extensively studied the mechanistic involvement of adrenals in glucotoxicity of OPI in rats mainly under acute and short-term exposure regimes. The rationale for studying adrenal involvement emerged from the typical hyperglycemic behaviour of single dose (oral) of two OPI-acephate and monocrotophos. Single dose of acephate and monocrotophos elicited rapid and transient hyperglycemia after administration. Both OPI were administered to overnight-fasted rats at 1/10 doses of their LD_{50} (LD50; acephate-1400mg/kg b.w., monocrotophos-18mg/kg b.w.). As depicted in **Fig. 4**, both acephate and monocrotophos induced reversible hyperglycemia with peak occurring at 2h after exposure. Acephate induced peak hyperglycemia at 2h (87%), which tended to normalize thereafter and attained near-control values at 8h after administration (Joshi & Rajini, 2009). Similarly, monocrotophos also induced rapid hyperglycemia with peak occurring at 2h (103%). Interestingly, monocrotophos induced hyperglycemia exhibited steep reversibility compared to acephate, with normalization occurring at 6h (Joshi & Rajini, 2010). This trend observed in the present study is consistent with other reports, which demonstrated reversible hyperglycemia in experimental animals following OPI administration. While Malathion has been reported to cause reversible hyperglycemia in rats (Gupta, 1974; Rodrigues et al., 1986; Seifert, 2001; Lasram et al., 2008), acute exposure to diazinon induced reversible hyperglycemia in mice (Seifert, 2001).

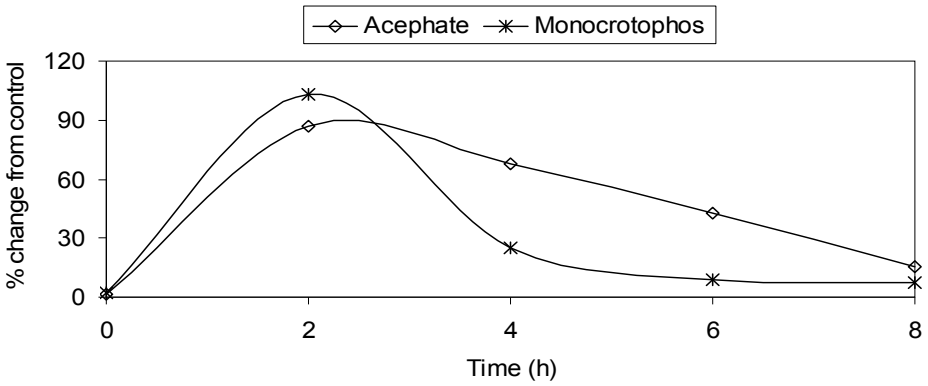

Fig. 4. Time-course of blood glucose levels in rats administered a single oral dose of acephate (140mg/kg b.w.) and monocrotophos (1.8mg/kg b.w.).

Based on the above results, we reasoned that the reversible hyperglycemia could be triggered by transient changes in the hormonal milieu of glucose homeostasis. Adrenals are an important part of the endocrine system and play a key role in glucose homeostasis by secreting glucocorticoid and amine hormones. Glucocorticoid hormones (GCs) (mainly cortisol in man and corticosterone in rodents) are secreted by the adrenal cortex under the control of hypothalamic-pituitary-adrenal axis. Glucocorticoid hormones, along with other key hormones, act to maintain blood glucose levels within narrow limits (Andrews & Walker, 1999). GCs, glucagon and epinephrine raise blood glucose by inhibiting glucose

uptake in the periphery and stimulating hepatic glucose release. Hepatic gluconeogenesis serves as the main source of hepatic glucose production during state of prolonged fasting and contributes significantly to development of diabetes mellitus (Pilkis & Granner, 1992). GCs facilitate gluconeogenesis as they exert permissive effect on the process by transcriptional activation of key enzymes of gluconeogenesis viz., glucose-6-phosphatase (G6Pase) (Argaud et al., 1996), phosphoenolpyruvate carboxykinase (PEPCK) (O'Brien et al., 1990) and tyrosine aminotransferase (TAT) (Ganss et al., 1994). Increased glycogenolysis and gluconeogenesis appear to be the two chief mechanisms underlying OPI-induced hyperglycemia. Fenitrothion-induced increase in blood glucose in *S. mossambicus* was associated with decreased hepatic glycogen (Koundinya & Ramamuthi, 1979) and sub chronic exposure of rats to acephate, which caused slight increase in blood glucose also caused depletion of liver glycogen in rats (Deotare & Chakrabarthi, 1981). Abdollahi et al. (2004a) reported increased activity of GP and phosphoenolpyruvate carboxykinase (PEPCK) following sub chronic exposure to Malathion. Acute exposure to diazinon has been shown to cause depletion of liver glycogen with increased activity of glycogen phosphorylase, and also increased activities of gluconeogenesis enzymes in liver (Matin et al., 1989). Valexon is reported to have increased the activity of G6Pase in liver of rats (Kuz'minskaia et al., 1978).

OPI and other AChE inhibiting organophosphate compounds exert strong influences on functioning of hypothalamic-pituitary-adrenal (HPA) axis, leading to increased circulating levels of corticosteroid hormones in vivo. This is particularly true in the case of acute exposure to AChE inhibiting compounds. Studies have shown elevated corticosteroid hormones levels in response to AChE-inhibiting compounds and role of AChE inhibition in the phenomenon. Single dose of Chlorfenvinphos, acephate and methamidophos have been demonstrated to elevate circulating levels of corticosterone and aldostserone after administration of a single dose (Osicka-Kaprowska et al., 1984; Spassova et al., 2000). Soman has been reported to increase plasma corticosterone levels in rodent models (Hudon & Clement, 1986; Fletcher et al., 1998). More importantly, the stressogenic potential (hypercorticosteronemia and induction of liver tyrosine aminotransferase activity) of soman was effectively abrogated by reactivators of inhibited acetylcholinesterase (Kassa, 1995 & 1997). Similarly, stressogenic potential of Cyclohexylmethyl phosphonofluoridate (AChE inhibitor) has been reported to be eliminated by HI-6 (AChE reactivator) (Kassa & Bajgar, 1995). Thus, it is clearly evident that AChE-inhibiting OPI elicit hyper stimulation of adrenal functions, leading to induction of gluconeogenesis enzymes in liver.

Based on the time-course of reversible hyperglycemia induced by acephate and monocrotophos, further experiments were carried out to investigate the adrenal effects of OPI and its role in the ensuing hyperglycemia. We assessed the effects of 2 or 6h exposure to either acephate (oral) or 2 or 4h exposure to monocrotophos (oral) on plasma corticosterone, adrenal cholesterol, blood glucose, key liver gluconeogenesis enzymes (G6Pase and TAT) and hepatic glycogen content in rats. Interestingly, we observed that both acephate and monocrotophos induced strong hypercorticosteronemia with concomitant hyperglycemia and induction of liver gluconeogenesis enzyme activities. Further, hypercorticosteronemia was associated with decrease in adrenal cholesterol pools (effect of monocrotophos on adrenal pools described in the section on 'comparison between single and repeated dose effects'), which is the precursor for corticosterone synthesis (**Table 3 & 4**). Depletion in adrenal cholesterol pools may therefore be attributable to increased synthesis and secretion of corticosterone. Interestingly, both OPI did not cause depletion in hepatic glycogen content. At time points that represented normalization of blood glucose levels, there was

phenomenal increase in liver glycogen levels. The data presented above clearly demonstrates co-existence of hypercorticosteronemia and induction of liver gluconeogenesis enzyme activities with hyperglycemia in OPI treated rats, indicating that OPI may trigger induction of liver gluconeogenesis machinery as result of hypercorticosteronemia, leading to hyperglycemia.

	At time interval after administration		
	0h	2h	6h
Plasma corticosterone *	30.9±3.4[a]	55.0±2.5[b]	44.0±2.7[b]
Adrenal cholesterol**	26.5±1.4[a]	15.6±0.56[b]	12.5±0.47[b]
Blood glucose ***	101.6±4.6[a]	182.4±5.2[b]	142.7±5.2[c]
Liver G6Pase#	90.14±4.38[a]	171.93±5.61[b]	112.84±4.18[c]
Liver TAT ##	14.28±1.34[a]	26.31±0.87[b]	23.7±0.48[b]
Hepatic glycogen$	316.2±34.90[a]	325.3±29.12[a]	1145.0±27.92[b]

*(*Joshi and Rajini, 2009*)*

Data analyzed by ANOVA followed by Tukey Test (n=6)
* µg/dl; ** mg/g tissue; *** mg/dl
# glucose-6-phosphatase (nmol/min/ mg protein);
tyrosine aminotranferase (nmol/min/mg protein); $ µg/g tissue

Table 3. Biochemical effects of acephate (140mg/kg b.w.) in rats

	At time interval after administration		
	0h	2h	4h
Plasma corticosterone *	36.62±1.2[a]	73.82±3.8[b]	45.65±1.8[a]
Blood glucose **	95.2±1.8[a]	194.8±3.7[b]	121.3±1.9[c]
Liver TAT #	15.86±0.8[a]	32.27±1.2[b]	26.87±1.8[c]
Hepatic glycogen##	213.8±49.2[a]	216.4±21.1[a]	925.7±27.6[b]

*(*Joshi and Rajini, 2010*)*

Data analyzed by ANOVA followed by Tukey Test (n=6)
* µg/dl; ** mg/g tissue ; # tyrosine aminotranferase (nmol/min/mg protein); ## µg/g tissue

Table 4. Biochemical effects of monocrotophos (1.8mg/kg b.w.) in rats

Indeed, role of adrenals in glucotoxicity of OPI has been explored earlier. Matin et al., (1989) earlier demonstrated that single dose diazinon (OPI) caused hyperglycemia and induction of liver gluconeogenesis enzymes in normal rats while these changes did not manifest in adrenalectomized rats, indicating the involvement of adrenals in the glucotoxicity of diazinon. Our attempts to study the adrenal and glycemic effects of acephate and monocrotophos revealed that two compounds, which exhibit anticholinesterase property, elicited similar effects. Thus, the effects raised question whether the adrenal and glycemic effects are mediated through the anticholinesterase property of OPI. To address the question, we studied the extent of AChE inhibition elicited by monocrotophos at 2 and 4h

after administration. Influence of cholinergic antagonists was investigated at 2h after administration on stressogenic (hypercorticosteronemia and induction of liver TAT activity) and hyperglycemic potential of monocrotophos. For the purpose of mechanistic investigations, we employed two muscarinic cholinergic antagonists- atropine sulphate, a general ACh receptor antagonist that can pass through blood brain barrier (BBB) (Guarini et al., 2004) and methyl atropine nitrate, which is a peripherally active antagonist that does not pass through blood BBB (Pavlov et al., 2006). Both antagonists were administered at 30μmols/kg b.w 3-5 min before monocrotophos (1/10 LD50).

Monocrotophos elicited significant inhibition of AChE activity (>50%) in brain, adrenals and liver at both 2 and 4 h after exposure (**Fig. 5A**). Of the organs studied, maximum inhibition of AChE activity was evident in brain (84 and 78% at 2 and 4 h respectively) while the enzyme activity in adrenals was inhibited to 32 and 34% of control activity at 2 and 4 h after exposure respectively. Similarly, monocrotophos administration reduced liver AChE activities to 47 and 46% of control at 2 and 4 h after exposure respectively. More importantly, we did not observe any spontaneous reactivation of inhibited AChE activity at 4h after administration, which is an important feature of the enzymes' behavior (Reiner and Aldridge, 1967; Reiner, 1971). This indicates that, while hyperglycemic potential of monocrotophos in rats may be a result of its anticholinesterase potency, the reversibility of hyperglycemia is not a consequence of spontaneous reactivation of the enzyme. Reversibility of hyperglycemia may hence be a consequence of counter-regulatory mechanism as reflected by glycogen deposition at 4h after administration. Increase in glycogen content upon 4h exposure is a clear indication of mobilization of glucose into glycogen synthesis pathway as a measure to overcome hyperglycemia.

We also observed that both cholinergic antagonists were potent in offering protection against stressogenic and hyperglycemic potential of monocrotophos. Administration of monocrotophos elicited significant hyperglycemia (103%) (**Fig. 5B)**. Pre- treatment of rats with atropine sulfate (106.04 ±1.83 compared to 191.82 ±7.59 mg/dl of monocrotophos alone) and atropine methyl nitrate (123.49 ±4.12 compared to 191.82 ±7.59 mg/dl of monocrotophos alone) offered significant protection against hyperglycemia induced by monocrotophos. It has been earlier demonstrated that diazinon-induced hyperglycemia was mediated by AChE inhibition, as revealed by protective effects of pralidoxime (AChE reactivator) (Seifert, 2001). Monocrotophos-induced hypercorticosteronemia (112%) was effectively prevented by cholinergic antagonists (**Fig. 5C)**. Pre-treatment of rats with atropine sulfate (33.98 ±2.89 compared to 76.63 ±1.76 μg/dl of monocrotophos alone) and atropine methyl nitrate (44.67 ±1.64 compared to 76.63 ±1.76 μg/dl of monocrotophos alone) offered significant protection against hypercorticosteronemia induced by monocrotophos. Monocrotophos induced a marked increase in the TAT activity in liver (107%) (**Fig. 5D**). Pre-treatment of rats with atropine sulfate (20.42 ±1.70 compared to 33.38 ±1.09 nmol/min/mg protein) and atropine methyl nitrate (22.39 ±0.79 compared to 33.38 ±1.09 nmol/min/mg protein) offered significant protection against induction of TAT activity. These results clearly indicated that both physiological stress (hypercorticosteronemia and induction of liver TAT activity) and hyperglycemia manifest as a consequence of peripheral muscarinic cholinergic stimulation. Corticosterone exerts hyperglycemic action by up-regulation of gluconeogenesis machinery. Hence, hypercorticosteronemia and induction of liver TAT (gluconeogenesis enzyme) activity accompanying hyperglycemia raises a question whether hypercorticosteronemia is responsible hyperglycemia in monocrotophos-treated rats.

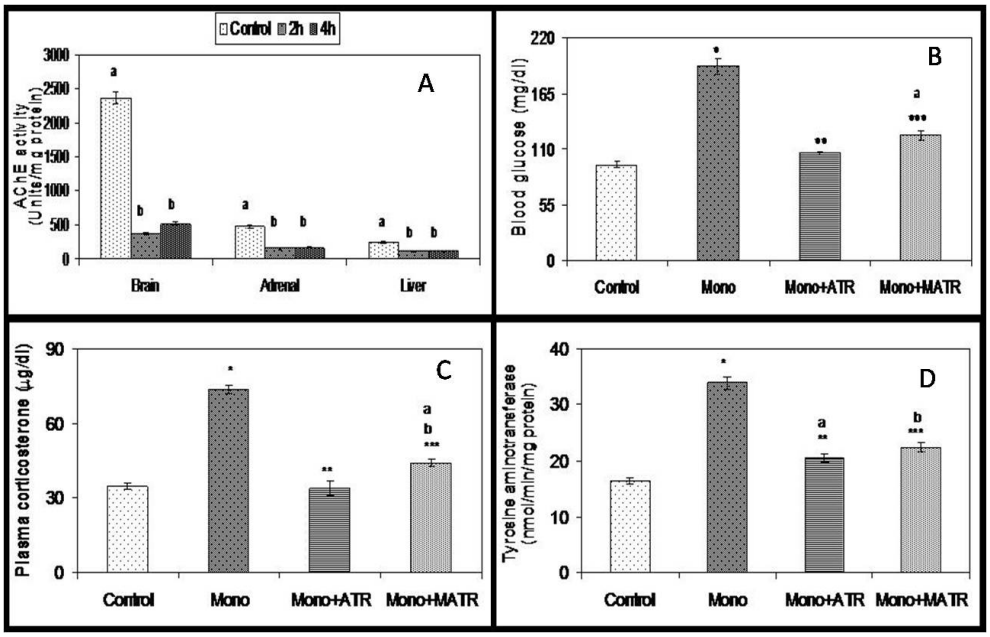

Fig. 5. Protective effects of atropine (ATR) and methyl atropine (MATR) against stressogenic and hyperglycemic potential of monocrotophos (Mono) *(*Joshi and Rajini, 2010*).*

Acetylcholine exerts strong influence on functioning of hypothalamus-pituitary-adrenal (HPA) axis. Acetylcholine has been found to increase corticotrophin releasing hormone (CRH) activity of hypothalamus *in vitro* as measured by effect on corticosteroidogenesis, an effect that was antagonized by atropine (Bradbury et al., 1974). ACh has also been shown to increase secretion of immunoreactive CRH from hypothalamus *in vitro* (Calogero et al., 1988), an effect that was antagonized by ACh receptor antagonists, atropine (muscarinic) and hexamethonium (nicotinic). Given the importance of ACh in excitation of HPA axis, assessment of cholinergic stress in activation of HPA axis in monocrotophos treated rats becomes important. The importance of ACh in functioning of HPA axis is further exemplified by the fact that muscarinic receptor agonists such as carbachol (Bugajski et al., 2002) and arecoline (Calogero et al., 1989) were found to increase ACTH and corticosterone *in vivo*. More importantly, the agonist induced increase in ACTH and corticosterone was antagonized by atropine (Bugajski et al., 2002), suggesting role for muscarinic ACh receptor in regulation of HPA axis. Role of anticholinesterase properties of organophosphate compounds in activation of HPA axis is demonstrated by studies showing elimination of stressogenic activity of cyclohexyl methyl phosphonofluoridate (as measured by plasma corticosterone and liver tyrosine aminotransferase activity) by HI-6, a cholinesterase reactivator that sufficiently reactivated inhibited AChE in brain and diaphragm (Kassa & Bajgar, 1995) and protection offered by atropine against diisopropylfluorophosphate induced increase in corticosterone levels (Smallridge et al., 1991). These studies clearly show the influence of ACh and involvement of muscarinic receptors in functioning of HPA axis.

From our data on influence of cholinergic antagonists on stressogenic and hyperglycemic potential of monocrotophos, it could be hypothesized that muscarinic cholinergic stress

triggers hypercorticosteronemia, which may lead to induction of liver gluconeogenesis and hyperglycemia. However, experiments conducted with glucocorticoid receptor and adrenergic receptor antagonists revealed that hyperglycemia in mediated by adrenergic mechanisms while hypercorticosteronemia leads to only induction of liver TAT activity (data not shown). Further, we observed that monocrotophos-induced hyperglycemia was completely abolished by a gluconeogenesis inhibitor (data not shown). This establishes that physiological stress and hyperglycemia manifest in monocrotophos treated rats as independent consequence of peripheral cholinergic stress.

We further compared the effects of monocrotophos on adrenal functions and glycemic control in rats following single and repeated doses. Comparison was made between the effects of a single dose (measured 2h after administration) and that of 5 or 10 doses (one dose per day, measured 2h after last dose). In both cases, the oral dose of 1.8mg/kg b.w. was employed for the purpose of comparison. Interestingly, we observed that effects single dose of monocrotophos on adrenal functions and glycemic control was more severe than that of repeated doses. Single dose of monocrotophos elicited hypercorticosteronemia (114%) with concomitant decrease in adrenal cholesterol (33%). These adrenal effects of single dose were accompanied with hyperglycemia (109%) and induction of liver tyrosine aminotransferase activity (113%). However, repeated administration of monocrotophos for 5 or 10days resulted in blunting of responses. In case of repeated exposure, increase in corticosterone was 76 and 67% respectively in 5 and 10d exposure groups with 18 and 13% decrease in adrenal cholesterol. Similarly repeated administration elicited marginal increase in blood glucose (39 and 32%) and induction of liver TAT activity (56 and 61%) (**Fig. 6**).

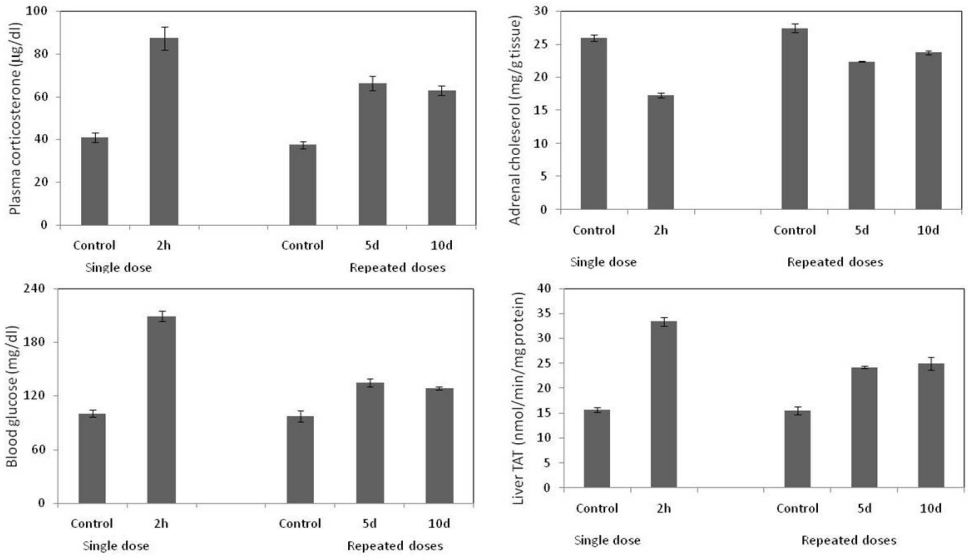

Fig. 6. Adrenal and glycemic effects of monocrotophos.

The above data clearly shows that repeated administration results in blunting of responses. This indicates that multiple administrations are associated with onset of some sort of resistance to the action of OPI. Development of tolerance to cholinesterase inhibitors during

multiple administrations is a well documented phenomenon (Brodeur and DuBois, 1964; McPhillips, 1969; Sterri et al., 1980). Tolerance to the elevation of plasma corticosterone by DFP was reported to develop during repeated administration (Kokka et al., 1985). Several studies suggest that cholinergic receptors could be involved in onset of tolerance to OPI, which may be mediated by events such as down regulation of muscarinic receptors (Costa et al., 1982a&b). Tolerance to the toxic effects of dilsulfoton during multiple exposures has been attributed to reduced muscarinic receptor binding in tissues of rats tolerant to the insecticide (Schwab et al., 1981). Blunted responses observed by us in case of repeated administration of monocrotophos may be attributed to tolerance mechanisms such as down regulation of muscarinic receptors. One mechanism that may be responsible for development of resistance is increased blood insulin levels. Comparison of effects of acute and repeated doses of monocrotophos on plasma insulin levels, however, needs to be done. Such a hyperinsulinemic response has been reported in case of exposure to malathion. While malathion caused increase in blood glucose and insulin levels after single exposure and continued dietary administration for 4 weeks, the degree of hyperinsulinemia was markedly greater in dietary group (Panahi et al., 2006). Thus, repeated administration of organophosphorus insecticides leads to blunting of responses. Although blunted, these responses still represent a great threat to euglycemic balance. This is particularly true in the case of constant state of hypercorticosteronemia. This has propensity to affect skeletal muscle glucose metabolism and long-term impairments in such mechanisms may lead to long lasting dysregulation in glucose homeostasis.

3.3 OPI act as pre-disposing factors for onset of diabetes?

Based on our comprehensive studies described above, we have proposed a scheme on the mechanism/s through which OPI might regulate/ disrupt glucose homeostasis (**Fig. 7**). Oxidative stress in pancreatic milieu and glucose intolerance, up regulated gluconeogenesis machinery and hyperglycemia are critical factors in diabetes etiology. With the ability to induce the above-mentioned dysregulations, OPI may have far reaching consequences on diabetic outcomes. This may be a more pertinent issue in the present times since diabetes is fast emerging as a major wide spread disorder that threatens human life. With this realization, our laboratory has also committed to investigate if OPI act as predisposing/aggravating factors for onset or progression of diabetic condition.

We observed that dichlorvos (DDVP) treated rats showed higher (22%) levels of blood glucose compared to normal control rats while as expected, rats injected with the diabetogenic agent, Streptozotocin (STZ) alone also showed elevated (37%) level of blood glucose. However, blood glucose levels of DDVP pre-treated rats administered STZ showed relatively higher blood glucose level compared to all the groups. Liver glycogen levels were significantly lower in rats administered either DDVP (18%) or STZ (19%) alone while, rats administered DDVP followed by STZ revealed further lower levels of glycogen (46 %) **(Table 5)**. Further, we also observed that DDVP pre-treatment resulted in more sever oxidative stress in STZ treated rats. ROS levels were significantly elevated in STZ (40%) and DDVP (55%) groups compared to 'untreated control' group. However, ROS levels were markedly higher (81.23 ± 6.52 pmole DCF/min/mg protein) in 'DDVP+STZ' group of rats. Pancreas of rats administered with either DDVP or STZ alone showed marginally higher levels of the lipid peroxides compared to that in 'untreated controls' while, the levels of lipid peroxides generated in pancreas of 'DDVP+STZ' rats showed

significant increase (110%) compared to all other groups. Pancreatic reduced glutathione level in 'DDVP+STZ' rats was significantly lower (37%) while, rats administered with DDVP or STZ alone also had significantly lower levels of GSH, although to a lesser extent (**Table 6**). These results clearly demonstrate that OPI act as pre-disposing factor for diabetes as reflected by higher degree of glucotoxicity of STZ (subdiabetogenic dose) in DDVP treated rats.

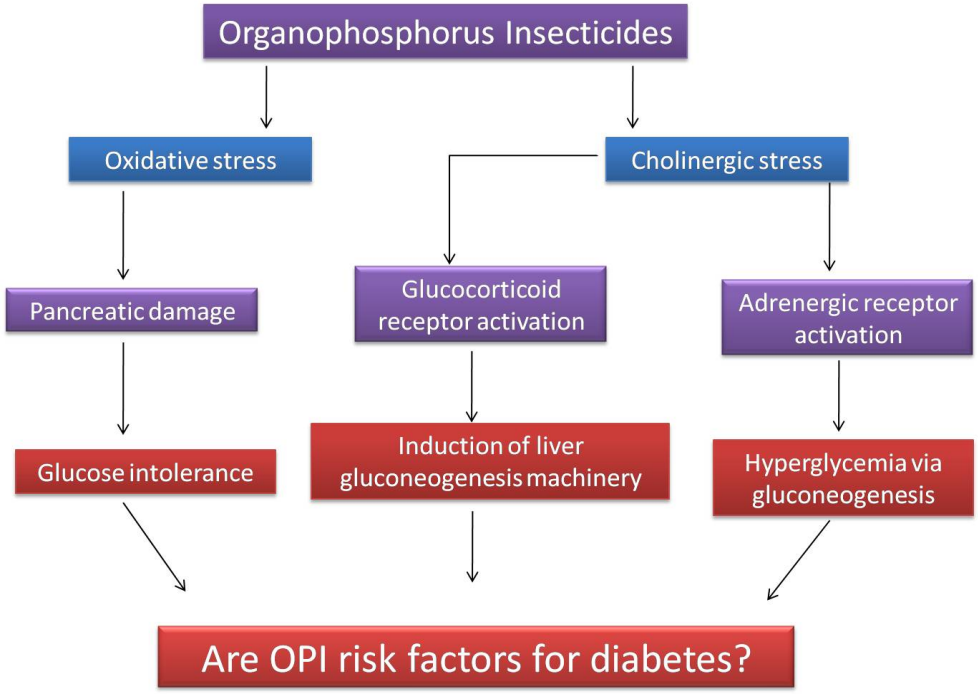

Fig. 7. Proposed scheme for OPI-induced alterations in glucose homeostasis.

Generally, an acute intraperitoneal dose of 40–60 mg/kg b.w is employed to induce significant hyperglycemia in rats. For the present study, we employed a lower dose of 25 mg/kg b.w ('sub-diabetogenic dose') in order to examine if pre-treatment with DDVP renders these rats more susceptible to hyperglycemia. Experimental regime began with two groups with 12 rats each-control and DDVP-treated group. The DDVP-treated group animals were orally administered daily DDVP at 20mg/kg b.w/d (corresponding to 1/5 of LD50 value: 100 mg/kg b.w, determined in a preliminary study) for 10 d. After 10 days, rats of the control group were further divided into two sub groups of six animals each ; the first sub group served as control ('untreated control'), while the second sub group of rats was intraperitoneally injected streptozotocin (STZ, 25 mg/kg b.w.) ('STZ'). The group of rats administered with DDVP was also divided into two sub groups; the first sub group of rats served as DDVP control ('DDVP'), while the second sub group of rats was injected with streptozotocin (i.p, 25mg/kg b.w.) ('DDVP+STZ').

Group	Blood glucose[1]	Liver glycogen[2]
CONTROL	$113.53^{a} \pm 2.31$	$41.55^{c} \pm 2.01$
DDVP	$138.37^{b} \pm 4.17$	$34.20^{b} \pm 1.42$
STZ	$155.03^{c} \pm 5.09$	$33.34^{b} \pm 2.23$
DDVP+STZ	$188.99^{d} \pm 4.44$	$22.62^{a} \pm 3.52$

[1]mg/dl; [2]mg/g tissue; Values are mean ± SEM (n=6); Mean in the same column with different superscript differ significantly ($p<0.05$)

Table 5. Blood glucose and liver glycogen levels in rats treated with DDVP ± STZ (i.p , 25 mg/kg b.w).

Group	ROS[1]	TBARS[2]	GSH[3]
Untreated control	$38.76^{a} \pm 4.04$	$242.76^{a} \pm 19.18$	$1.07^{c} \pm 0.03$
DDVP	$60.18^{b} \pm 4.59$	$294.94^{a} \pm 10.65$	$0.83^{b} \pm 0.01$
STZ	$54.27^{b} \pm 2.89$	$283.63^{a} \pm 7.27$	$0.78^{b} \pm 0.02$
DDVP+STZ	$81.23^{c} \pm 6.52$	$389.38^{b} \pm 38.47$	$0.67^{a} \pm 0.02$

[1]pmol DCF/min/mg protein; [2]nmol/g tissue; [3]mg/g tissue
Values are mean ± SEM (n=6); Mean in the same column with different superscript differ significantly ($p<0.05$)

Table 6. Oxidative stress parameters in pancreas of rats treated with DDVP ± STZ (i.p , 25 mg/kg b.w).

3.4 Do OPI aggravate diabetic outcomes?

To address this question, we investigated the diabetic outcomes in rats experimentally rendered diabetic and post-treated with monocrotophos. Rats were rendered diabetic with an acute dose (60 mg/kg b.w, i.p) of streptozotocin. Monocrotophos was orally administered at a sublethal dose (1/20 LD_{50}, 0.9 mg /kg b.w./d, 5 days) to both normal and diabetic rats. We observed that monocrotophos *per se* moderately increased (25%) the blood glucose levels in normal rats, but significantly aggravated the hyperglycemic outcome in diabetic rats (56% above diabetic rats). We observed the typical lipid profile alterations among diabetic rats characterized by increase in total cholesterol and triglycerides (TG) in serum. While monocrotophos did not impact lipid profile *per se*, its interaction with diabetic component resulted in severe alterations lipid profile that reflected in phenomenal increase in serum triglyceride. Such augmented impairments may have high bearing on cardiovascular health since the cardiovascular risk index was alarmingly high among diabetic rats treated with monocrotophos (**Fig. 8**). Further, monocrotophos resulted in higher degree of hepatic and renal toxicity as reflected by alterations in serum transaminase activities and blood urea nitrogen values respectively (**Table 7)**.

Further, it is now established that oxidative stress in an important consequence of diabetic condition and plays as an important pathophysiological factor in progression of diabetic complications (Maritim, 2003). We observed that STZ-induced diabetes was associated with increased lipid peroxidation (122%), depletion of reduced glutathione (10%) and alterations in activities of two important antioxidant enzymes (superoxide dismutase and catalase) in kidney. Monocrotophos further deteriorated oxidative impairments in kidney as evidenced by further increase in lipid peroxidation (170%) and depletion of reduced glutathione (18%) content. Diabetes was associated with marginal decrease in superoxide dismutase activity in kidney, which was further reduced by monocrotophos treatment (35%) (**Fig. 9**).

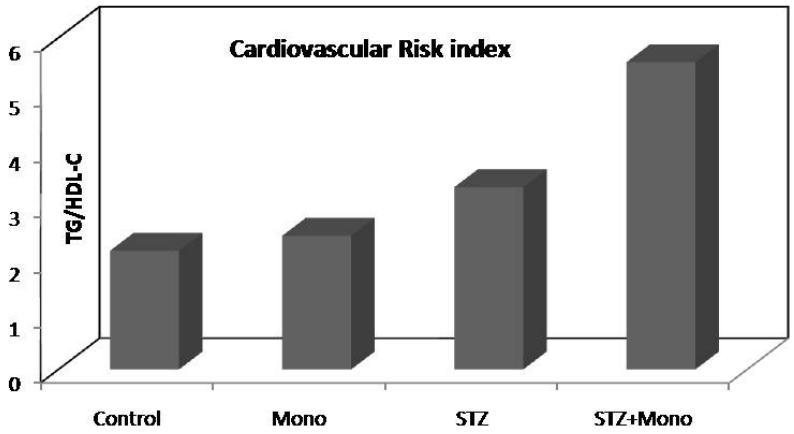

Fig. 8. Effect of repeated oral doses of monocrotophos at 1/20 LD_{50} (0.9 mg/kg b.w) on cardiovascular index in control and diabetic rats (Begum and Rajini, 2011a).

	Control	Mono	STZ	STZ+Mono
Blood glucose	101.58 ±	126.91±	382.71 ±	597.94 ±
(mg/dl)	1.4	8.9	14.0^{ab}	12.5^{cde}
TC	38.00 ±	41.92 ±	50.45 ±	50.42 ±
(mg/dl)	2.1	1.9	1.6^{a}	1.2^{a}
HDL-C	31.09 ±	32.68 ±	37.84 ±	35.48 ±
(mg/dl)	1.2	1.7	1.4	1.3
TG	66.74 ±	78.12 ±	125.44 ±	193.52 ±
(mg/dl)	3.5	6.9	9.2^{a}	19.4^{bcd}
BUN	33.08 ±	51.88 ±	71.18 ±	78.05 ±
(mg/dl)	5.6	8.2	10.1^{a}	5.2^{b}
SC	0.63 ±	0.72 ±	0.81 ±	0.84 ±
(mg/dl)	0.06	0.1	0.1	0.04
Serum ALT	66.19 ±	72.24 ±	80.03 ±	120.77 ±
(U/L)	2.0	0.7	2.4	9.2^{abc}
Serum AST	126.57 ±	156.00 ±	199.78 ±	341.55 ±
(U/L)	0.2	18.7	14.2^{de}	8.5^{abc}

(Begum and Rajini, 2011a)

Data analyzed by Tukey's HSD test; Mean ± SEM (n=4)
TC: Total cholesterol; HDL-C: High-density lipoprotein ; TG: Triglyceride; BUN: Blood Urea Nitrogen; SC: Serum creatinine

Table 7. Effect of repeated oral doses of monocrotophos at 1/20 LD_{50} (0.9 mg/kg b.w) on blood glucose, lipid profile and hepatic and renal damage markers in serum in control and diabetic rat.

Our work on interaction of OPI with diabetic component clearly shows that OPI can act as both predisposing and aggravating factors for diabetes. The inference becomes an important consideration to be made as the modern world is facing an escalating situation of alarming increase in the incidence of diabetes. Our study employed a low dose of monocrotophos,

which *per se* did not interfere with lipid profile in rats, yet causing augmentation of alteration in lipid profile in diabetic rats. However, other studies have clearly demonstrated that several OPI cause alterations in lipid profile, particularly hypertriglyceridemia (Ryhänen et al, 1984; Ibrahim & El-Gamal, 2003; Rezg et al., 2010). Dyslipidemia or lipid abnormalities play an important role in the progression of diabetes (Goldberg, 2001) and these are characterized by lipid derangements including hypertriglyceridemia, low high-density cholesterol (HDL-C), and a high concentration of small dense low-density lipoprotein (LDL) particles. Further, a state of elevated hypertriglyceridemia is commonly associated with insulin resistance and represents a valuable clinical marker of the metabolic syndrome (Grundy et al., 1999). Propensity of OPI to induce hypertriglyceridemia coupled with their permissive effects of gluconeogenesis in liver creates a serious threat to glucose homeostasis.

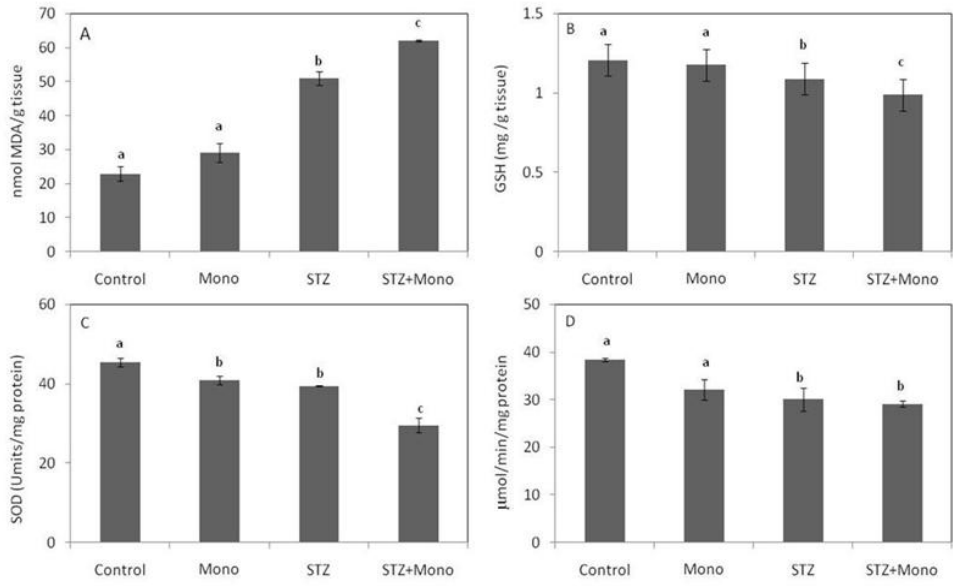

Fig. 9. Effect of repeated oral doses of monocrotophos at 1/20 LD_{50} (0.9 mg/kg b.w) on oxidative balance in kidney of control and diabetic rats (Begum and Rajini, 2011b).

4. Conclusion

Given the status of OPI as environmental pollutant with residues being detected in biosphere around, which are now being shown to make it into human body, it is almost certain that OPI will interact with etiological factors of diabetes at toxicologically significant levels. Interaction of living system with OPI may have severe two-way impact on glycemic control. As documented facilitators of hepatic glucose output via glycogenolysis and gluconeogenesis, OPI are most likely to elicit hyperglycaemic responses in humans during exposure. Further, OPI may also affect the responsiveness to human system to insulin via

multiple mechanisms, causing predisposition to diabetes. From our studies, it is also clear that OPI may also act to augment diabetic outcomes. In most societies, large sections of populations are subject to diabetes risk factors such as unhealthy diet patterns, lack of physical exercise and obesity. With such high odds of risk factors, the burden of constant exposure to OPI (as environmental pollutants) could be a silent aggravating factor that is causing increase in incidence of diabetes.

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6. References

Abdollahi, M., Donyavi, M., Pournourmohammadi, S., & Saadat M. (2004a). Hyperglycemia associated with increased hepatic glycogen phosphorylase and phosphoenol pyruvate carboxykinase in rats following sub-chronic exposure to malathion. *Comparative Biochemistry & Physiology (C)*, 137, 343-347.

Abdollahi, M., Ranjbar, A., Shadnia, S., et al. (2004b). Pesticides and oxidative stress; review. *Medical Science Monitor*, 10, 141-147.

Abou-Donia, M. (2003). Organophosphorus Ester-Induced Chronic Neurotoxicity. *Archives of Environmental Health*, 58, 484-497.

Akhgari, M., Abdollahi, M., Kebryaeezadeh, A., et al. (2003). Biochemical evidence for free radical-induced lipid peroxidation as a mechanism for sub chronic toxicity of malathion in blood and liver of rats. *Human & Experimental Toxicology*, 22, 205-211.

Andrews, RC., & Walker, BR. (1999). Glucocorticoids and insulin resistance: old hormones, new targets. *Clinical Science,* 96, 513-523.

Argaud. D., Zhang, Q., Malatra, S., et al. (1996). Regulation of rat liver glucose-6-phosphatase gene expression in different nutritional and hormonal states. *Diabetes*, 45, 1563-1571.

Banerjee, BD., Seth, V., & Ahmed, RS. (2001). Pesticide-induced oxidative stress: perspective and trends. *Reviews on Environmental Health*, 16, 1-40.

Begum, K, & Rajini, PS. (2011a). Monocrotophos augments the early alterations in lipid profile and organ toxicity associated with experimental diabetes in rats. Pesticide Biochemistry & Physiology, 99, 33-38.

Begum, K & Rajini, PS. (2011b). Augmentation of hepatic and renal oxidative stress and disrupted glucose homeostasis by monocrotophos in streptozotocin-induced diabetic rats. *Chemico Biological Interactions*, (In press).

Bradbury, MWB., Burden, J., Hillhouse, EW., & Jones, MT. (1974). Stimulation electrically and by acetylcholine of the rat hypothalamus in vitro. *Journal of Physiology*, 239, 269–83.

Brodeur, J., & DuBois, KP. (1964). Studies on the mechanism of acquired tolerance by rats O, O-diethyl S-2- (ehylthio) ethyl phosphorodithioate (Di-syston). *Archives of International Pharmacodynamics*, 149, 560-570.

Bugajski, J., Gadek-Michalska, A., & Bugajski, AJ. (2002). Effect of constitutive- and inducible-cyclooxygenase in the carbachol-induced pituitary-adrenocortical response during social stress. *Journal of Physiology & Pharmacology,* 53, 453-462.

Calogero AE, Kamilaris TC, Gomez MT, et al. (1989). The muscarinic cholinergic agonist arecoline stimulates the rat hypothalamic-pituitary-adrenal axis through a centrally-mediated corticotropin-releasing hormone-dependent mechanism. *Endocrinology*, 125, 2445-2453.

Calogero, AE., Gallucci, WT., Bernardini, R., et al. (1988). Effect of cholinergic agonists and antagonists on rat hypothalamic corticotropin-releasing hormone secretion in vitro. *Neuroendocrinology*. 47, 303-308.

Costa, LG., Schwab, BW., & Murphy, SD. (1982a). Differential alterations of cholinergic muscarinic receptors during chronic and acute tolerance to organophosphorus insecticides. *Biochemical Pharmacol*ogy, 31, 3407-3413.

Costa, LG., Schwab, BW., & Murphy, SD. (1982b). Tolerance to anticholinesterase compounds in mammals. *Toxicology*, 25, 79-97.

Deotare, ST., & Chakrabarti, CH. 1981. Effect of acephate (orthene) on tissue levels of thiamine, pyruvic acid, lactic acid, glycogen and blood sugar. *Indian Journal of Physiology & Pharmacology,* 25, 259-264.

Dressel, TD., Goodale, RL Jr, Zweber B, & Borner JW. 1982. The effect of atropine and duct decompression on evolution of diazinon-induced canine pancreatitis. *Annals of Surgery,* 195: 424-434.

Dressel, TD., Goodale, RL Jr., Ameson, MA., & Borner, JW. (1979). Pancreatitis as a complication of anticholinesterase insecticide intoxication. *Annals of Surgery*, 189, 199-204.

Eto, M. (1974). *Organophosphorus Pesticides: Organic and Biological Chemistry*, 387 pp, CRC Press, Cleveland, OH.

Fletcher, HP, Akbar, WJ, Peoples, RW, & Spratto, GR. (1998). Effect of acute soman on selected endocrine parameters and blood glucose in rats. *Fundamental & Applied Toxicology,* 11, 580-586.

Frick, TW, Dalo, S, O'Leary, JF, et al. (1987). Effects of insecticide, diazinon, on pancreas of dog, cat and guinea pig. *Journal of Environmental Pathology, Toxicology and Oncology,* 7, 1-11.

Fukuto, TR. (1990). Mechanism of action of organophosphorus and carbamate insecticides. *Environmental Health Perspectives,* 87, 245-254.

Galloway, T, & Handy R. (2003). Immunotoxicity of organophosphorus pesticides. *Ecotoxicology*, 12, 345-363.

Ganss, R, Weih, F, & Schutz G. (1994). The Cyclic Adenosine 3′5′-Monophosphate and the glucocorticoids-dependent enhancers are targets for insulin repression of tyrosine aminotransferase gene transcription. *Molecular Endocrinology*, 8, 895-903.

Gokalp, O, Buyukvanh, B, & Cicek E, et al. (2005). The effects of diazinon on pancreatic damage and ameliorating role of vitamin E and vitamin C. *Pesticide Biochemistry & Physiology*, 81, 123-128.

Goldberg, JI. (2001). Diabetic dyslipidemia: causes and consequences. *The Journal of Clinical Endocrinology & Metabolism,* 86, 965–971.

Grundy, SM. (1999). Hypertriglyceridemia, insulin resistance, and the metabolic syndrome. *American Journal of Cardiology*, 83, 25F-29F.

Guarini, S, Cainizzo, MM, Giuliani, D, et al. (2004). Adrenocorticotropin reverses hemorrhagic shock in anesthetized rats through the rapid activation of a vagal anti-inflammatory pathway. *Cardiovascular Research,* 63, 357-365.

Gupta, PK. (1974). Malathion induced biochemical changes in rats. *Acta Pharmacology et. Toxicology,* 35, 191-194.

Hsiao, CT, Yang, CC, Deng, JF, et al. (1996). Acute pancreatitis following organophosphate intoxication. *Journal of Toxicology Clinical Toxicology,* 34, 343-347.

Hudon, M, & Clement, JG. (1986). Effect of soman (pinacolyl methylphosphonofluoridate) on the blood levels of corticosterone and adrenocorticotropin in mice. *Candian Journal of Physiology & Pharmacology,* 64, 1339-1342.

Ibrahim, NA, El-Gamal, BA. (2003). Effects of diazinon, an organophosphate insecticide, on plasma lipid consitutents in experimental animals. *Journal of Biochemistry and Molecular Biology,* 36, 499–504.

Joshi AK, Rajini PS. 2010. Hyperglycemic and stressogenic effects of monocrotophos in rats: Evidence for the involvement of acetylcholinesterase inhibition. *Experimental & Toxicologic Pathology,* (In press).

Joshi, AK, & Rajini, PS. (2009). Reversible hyperglycemia in rats following acute exposure to acephate, an organophosphorus insecticide: role of gluconeogenesis. *Toxicology*, 257, 40-45

Kamath, V, Joshi, AKR, & Rajini, PS. (2008). Dimethoate induced biochemical perturbations in rat pancreas and its attenuation by cashew nut skin extract. *Pesticide Biochemistry & Physiology*, 90, 58-65.

Kamath, V, Rajini, PS. (2007). Altered glucose homeostasis and oxidative impairment in pancreas of rats subjected to dimethoate intoxication. *Toxicology*. 231, 137-146.

Kassa, J, & Bajgar, J. (1995). Comparison of the efficacy of HI-6 and obidoxime against cyclohexyl methyl phosphonofluoridate (GF) in rats. *Human & Experimental Toxicology*, 11, 923-928.

Kassa, J. (1995). Comparison of efficacy of two oximes (HI-6 and obidoxime) in soman poisoning in rats. *Toxicology,* 101, 167-174.

Kassa, J. (1997). Importance of cholinolytic drug selection for the efficacy of HI-6 against soman in rats. *Toxicology*, 116, 147-152.

Kokka, N, Lee, R, & Lomax P. (1985). Effects of organophosphae cholinesterase inhibitors on pituitary adrenal activity and thermoregulation. *In Fifth Annual Chemical Defense Bioscience Review*. 60.

Koundinya, PR, Ramamurthi, R. (1979). Effect of organophosphate pesticide (fenitrothion) on some aspects of carbohydrate metabolism in a freshwater fish, Saotherodon (Tilapia) mossamicus (Peters). *Experientia,* 35, 1632-1633.

Kuz'minskaia, UA, Bersan, LV, & Veremenko, LM. (1978). Activity of the indicator enzymes of liver subcellular structures with the prolonged administration of Valexon. *Vopr. Pitan*, 5, 48-51.

Lasram, MM, Annabi, AB, Rezg R, et al. (2008). Effect of short-time Malathion administration on glucose homeostasis in Wistar rat. *Pesticide Biochemistry & Physiology,* 92, 114-119.

Maritim, AC, Sanders, RA, Watkins (III), JB. (2003). Diabetes, Oxidative Stress, and Antioxidants: A Review. *Journal of Biochemical & Molecular Toxicology*, 17, 24-38.

Marsh, WH, Viekov, GA, & Conradi, EC. (1988). Acute pancreatitis after cutaneous exposure to an organophosphate insecticide. *American Journal of Gastroenterology,* 83, 1158-1160.

Mathur HB, Johnson S, Mishra R., et al. (2003). Analysis of pesticide residues in bottled water (Delhi Region). CSE Report.
http://www.cseindia.org/userfiles/Delhi_uploadfinal_sn.pdf

Mathur, HB, Agarwal, HC, Johnson, S, & Saikia, N. (2005). Analysis Of Pesticide Residues In Blood Samples From Villages Of Punjab. CSE Report.
http://www.cseindia.org/userfiles/Punjab_blood_report.pdf

Matin, MA, Khan, SN, Hussain K, & Sattar S. 1989. Effects of adrenalectomy on diazinon-induced changes in carbohydrate metabolism. *Archives of Toxicology,* 63, 376-380.

McPhillips, JJ. (1969). Altered sensitivity to drugs following repeated injections of a cholinesterase inhibitor to rats. *Toxicology and Applied Pharmacology,* 14, 67-73.

Meller, D, Fraser, I, & Kryger, M. (1981). Hyperglycemia in anticholinesterase poisoning. *Candian Medical Association Journal*, 124, 745-748.

Mollaoglu, H, Yilmaz, HR, Gokalp, O, & Altuntas I. (2003). Methidathion un pankreas uzerine erkileri: Vitamin E ve C nin rolu. *Van Tip Dergisi*, 10, 98-100.

Moore, PG, & James, OF. (1988). Acute pancreatitis induced by acute organophosphate poisoning.
Postgraduate Medical Journal, 57, 660-662.

Namba, T. (1971). Cholinesterase inhibition by organophosphorus compounds and its clinical effects. *Bulletin - World Health Organization,* 44, 289-307.

O'Brien, RM, Lucas, PC, Forest CD, et al. (1990). Identification of a sequence in the PEPCK gene that mediates a negative effect of insulin on transcription. *Science*. 249, 533-537.

Osicka-Kaprowska, A, Lipska M, Wysocka-Paruzewska, B. (1984). Effect of chlorfenvinphos on plasma corticosterone and aldosterone levels in rats. *Archives of Toxicolicology*, 55, 68-69.

Panahi,P, Vosough-Ghanbari, S, Pournourmohammadi, S, et al. (2006). Stimulatory Effects of Malathion on the Key Enzymes Activities of Insulin Secretion in Langerhans Islets, Glutamate Dehydrogenase and Glucokinase. *Toxicology Mechanisms & Methods,* 16, 161–167.

Pavlov, VA, Ochani M, Gallowitsch-Puetra, M, et al. (2006). Central muscarinic cholinergic regulation of the systemic inflammatory response during endotoxemia. *Proceedings of the National Academy of Sciences, USA,* 103, 5219-5223.

Pilkis, SJ, & Granner, DK. (1992). Molecular physiology of the regulation of hepatic gluconeogenesis and glycolysis. *Annual Review of Physiology,* 54, 885-909.

Rahimi, R, & Abdollahi, M. (2007). A review on the mechanisms involved in hyperglycemia induced by organophosphorus pesticides. *Pesticide Biochemistry & Physiology,* , 115-121.

Ranjbar, A, Pasalar, P, & Abdollahi, M. (2002). Induction of oxidative stress and acetylcholinesterase inhibition in organophosphorous pesticide manufacturing workers. *Human & Experimental Toxicology,* 21, 179-182.

Reiner, E, & Aldridge, WN. (1967). Effect of pH on inhibition and spontaneous reactivation of acetylcholinesterase treated with esters of phosphorus acids and carbamic acids. *Biochemical Journal,* 105: 171-179.

Reiner, E. (1971). Spontaneous reactivation of phosphorylated and carbamylated cholinesterase. *Bulletin of World Health Organization,* 44, 109-112.

Rezg, R, Mornagui, B, Benahmed, M, etal. (2010). Malathion exposure modulates hypothalamic gene expression and induces dyslipedemia in Wistar rats. *Food & Chemical Toxicology.* 48, 1473-1477.

Rodrigues, MA, Puga, FR, Chenker, E, & Mazanti, MT. (1986). Short-term effect of malathion on rats' blood glucose and on glucose utilization by mammalian cells in vitro. *Ecotoxicology & Environnemental Safety,* 12 , 110-113.

Ryhänen, R, Herranen, J, Korhonen, K, et al. (1984). Relationship between serum lipids, lipoproteins and pseudocholinesterase during organophosphate poisoning in rabbits. *International Journal Biochemistry.* 16: 687-690.

Sanghi, R, Pillai, MK, Jayalekshmi, TR, & Nair A. (2003). Organochlorine and organophosphorus pesticide residues in breast milk from Bhopal, Madhya Pradesh, India. *Human & Experimental Toxicology.* 22, 73-6.

Schwab, BW, Hand, H, Costa, LG, & Murphy, SD. (1981). Reduced muscarinic receptor binding in tissues of rats tolerant to the insecticide disulfoton. *Neurotoxicology.* 2, 635-47.

Seifert, J. (2001). Toxicological significance of the hyperglycemia caused by organo-phosphorous insecticides. *Bulletin of Environmental Contamination & Toxicology,* 67, 463-469.

Sevillano, S, de la Mano, AM, Manso, MA, et al. (2003). N-acetylcysteine prevents intra-acinar oxygen free radical production in pancreatic duct obstruction-induced acute pancreatitis. *Biochimica et Biophysica Acta,* 20, 177-184.

Smallridge, RC, Carrb, FE, & Feina, HG. (1991). Diisopropylfluorophosphate (DFP) reduces serum prolactin, thyrotropin, luteinizing hormone, and growth hormone and increases adrenocorticotropin and corticosterone in rats: Involvement of dopaminergic and somatostatinergic as well as cholinergic pathways. *Toxicology & Applied Pharmacology,* 180, 284-295.

Sogorb, MA, & Vilanova, E. (2002). Enzymes involved in detoxification of organophosphorus, carbamate and pyrethroid insecticides through hydrolysis. *Toxicology Letters,* 128, 215-228.

Soltaninejad, K, & Abdollahi, M. (2009). Current opinion on the science of organophosphate pesticide and toxic stress: A systematic review. *Medical Science Monitor,* 15, RA75-RA90.

Spassova, D, White, T, & Singh, AK. (2000). Accute effects of acephate and methamidophos on acetylcholinesterase activity, endocrine system, and amino acid concentrations in rats. *Comparative Biochemistry & Physiology Part (C),* 12, 79-89.

Sterri, SH, Lyngaas, S, & Fonnum, F. (1980). Toxicity of soman after repetitive injection of sublethal doses in rat. *Acta Pharmacology et Toxicology (Copenh).* 46, 1-7.

Sultatos, LG. (1994). Mammalian toxicology of organophosphorous pesticides. *Journal of Toxicology & Environmental Health*, 43, 271-289.

4

The Toxicity of Fenitrothion and Permethrin

Dong Wang, Hisao Naito and Tamie Nakajima
Nagoya University Graduate School of Medicine
Japan

1. Introduction

Fenitrothion: An organophosphorus insecticide, fenitrothion (*O*,*O*-dimethyl *O*-4-nitro-*m*-tolyl phosphorothioate; CAS No. 122-14-5), which is a yellow-brown liquid with an unpleasant odor at room temperature, was introduced in 1959 by both Sumitomo Chemical Company and Bayer Leverkusen, and later by American Cyanamid Company (Hayes, 1982; Hayes and Laws, 1990; Worthing and Walker, 1987).

Organophosphorus insecticides began with the massive development of agriculture and agribusiness after World War II. At that time, parathion, one of the famous organophosphorus insecticides, was used in large quantities for preventing rice-stem borer worldwide. However, because of the high acute toxicity, parathion was thought to be an extremely hazardous substance. In man, an oral dose of 3-5 mg/kg is usually fatal. The following case report additionally came across the high toxicity and persistence in humans (Clifford and Nies, 1989). A 25-year-old worker in a pesticide-formulating plant was contaminated after accidentally spilling a 76% parathion solution on his groin and scrotal areas. Although he showered and changed clothes immediately, the resulting nausea and diarrhea made him consult a doctor two days later. The worker placed the parathion-saturated uniform in a plastic bag to be burned. But the contaminated clothing was laundered, and then was used in succession by a second worker, who wore it until he had complaints similar to the first worker. The coveralls were again laundered and used by still a third intoxicated worker. Totally, three workers suffered from toxic reaction to parathion. This case shows the toxic nature of parathion and its persistence on clothing even after successive laundering. Moreover, Etzel et al. (1987) reported that 49 persons in Sierra Leone were acutely poisoned by parathion in May and June 1986, 14 of whom later died. The case-control study of the employed 21 cases and 22 household controls was undertaken to explore which factors were associated with the development of the symptoms such as excess salivation, excess tearing, increased urination, diarrhea, convulsions, and loss of consciousness. Each case and control were questioned about foods and beverages that had been consumed during the 4 hours before becoming ill (for cases) or on the day of a case's illness (for controls). The odds ratio of cases (12.7; 95% confidence interval (CI), 2.4-83.8) for taking bread was significantly increased, suggesting that cases were more likely than controls to have eaten bread within the 4 hours before becoming ill. In addition, when stratified by age, the odds ratio was far higher in children under 18 years (odds ratio, 21.7; 95% CI, 2.4-264.6) than adults (odds ratio, 2.3; 95% CI, 0.02-195.9). This may be due to the higher consumption of parathion based on body weight or higher susceptibility to the

insecticide in the former than the latter. Parathion was detected from residue floor on the truck that had brought the wheat flour from the milling factory to the general store where the baker purchased it, suggesting that the flour had been contaminated during transport. The authors estimated that 10-15 ml of parathion may have spilled onto a 22.5 kg bag of flour in the truck. Besides these, many cases of parathion intoxication have been reported to date (Aardema et al., 2008; Eyer et al., 2003; Hoffmann and Papendorf, 2006; Laynez et al., 1997).

In light of this background, fenitrothion was developed in place of parathion for its highly selective toxicity to insects over humans and animals. While the structure of fenitrothion is similar to that of parathion, its residual effects and acute toxicity are lower than parathion (Miyamoto, 1969). An oral LD50 of parathion is approximately 6 mg/kg for rats, against 330 mg/kg for fenitrothion, which is more rapidly broken down and does not persist in areas where they are used. Fenitrothion is effective against a wide range of pests on rice, cereals, fruits, vegetables, stored grains, cotton and forests, and also in public health programs for control of flies, mosquitoes and cockroaches. Fenitrothion is produced at the rate of 15,000 to 20,000 tons per year worldwide, and is available in emulsifiable concentrates, ultra-low-volume concentrates, powders, granules, dustable powders, oil-based sprays and in combination with other pesticides.

Permethrin: Permethrin (3-phenoxybenzyl (1*RS*,3*RS*;1*RS*,3*SR*)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate; CAS No. 52645-53-1) was first synthesized in 1973 and marketed in 1977 as a photostable pyrethroid (Elliott et al., 1973). Approximately 600 tons per year is at present used worldwide not only in agriculture but also in forestry, household settings, and public health programs.

Pyrethroids represented a major advancement as a high insecticide potential, but showed relatively low potential for mammals. Their development was especially timely with the identification of problems with DDT use. Pyrethroids consist first of identifying components of pyrethrum, which were extracted from East African chrysanthemum flowers, long known to have insecticidal properties. Pyrethrum rapidly knocks down flying insects, but has low mammalian toxicity and negligible persistence, which are good for the environment but yield poor efficacy when applied in the field. In the 1960s, 1st-generation pyrethroids, including bioallethrin, tetramethrin, resmethrin and bioresmethrin, were developed. They are more active than natural pyrethrum, but are unstable in sunlight. Then, permethrin, cypermethrin and deltamethrin were discovered as a 2nd generation of more persistent compounds. They are substantially more resistant to degradation by light and air, thus making them suitable for use in agriculture.

Permethrin is highly effective for protection of stored grains, cotton and other crops, and the control of body lice and household noxious insects. Technical products, which are a brown or yellowish-brown liquid, are a mixture of *cis* and *trans* isomers in the ratio of 40:60 or 25:75, and are available in emulsifiable concentrates, ultra-low-volume concentrates, wettable powders, and dustable powders.

2. Absorption, metabolism and excretion in laboratory animals and humans

In mammals, fenitrothion and permethrin are absorbed via gastrointestinal or respiratory tract and skin, and are rapidly metabolized and excreted.

Fenitrothion: After uptake into the body, fenitrothion is metabolized by hepatic cytochrome P450 (CYP) to form fenitrooxon, which is thought to have a higher potential acute neurotoxicity than the parent compound. Fenitrooxon is further metabolized to

dimethylphosphate and 3-methyl-4-nitrophenol (MNP) by paraoxonase 1 (PON1). MNP and methylphosphate are also produced by glutathione-S-aryltransferase (GST) and PON1. In another pathway, fenitrothion is directly metabolized to MNP and dimethylthiophosphate by PON1 or MNP and methylthiophosphate by GST and PON1 (Figure 1). Interestingly, its major metabolic route differs between mammals and birds as mentioned later. Most of the metabolites are excreted in urine within 24 hours in humans (Nosal and Hladka, 1968), and within 2-4 days in the rat, guinea-pig, mouse, and dog (Miyamoto et al., 1963; Miyamoto, 1964). Species and sexes differences are observed in the composition of the metabolites. MNP, which is also contained in diesel exhaust emissions, has potential adverse effects on the reproductive systems in mammals and birds. Fenitrothion at doses of 0.18 and 0.36 mg/kg per day was administered to 12 human volunteers for 4 days (Meaklim et al., 2003). Pharmacokinetic parameters could only be determined at the high dosage, because the blood levels of fenitrothion at the low dosage were below the detectable level. Fenitrothion concentrations showed a wide range of interindividual variability, with peak blood levels achieved 1-4 hours after dosing, and the half-life ranged from 0.8 to 4.5 hours. Serum concentrations of fenitrothion were measured in 15 patients after acute fenitrothion intoxication, who admitted to the hospital 0.5-12 hours after the ingestion of 5-50 g fenitrothion (Koyama et al., 2006). The serum fenitrothion concentrations ranged from undetectable (< 0.01 μg/ml) to 9.73 μg/ml. Serum fenitrothion concentrations were less than 7 μg/ml in the patients with mild intoxication, while in the severe cases, the levels were more than 7 μg/ml. The elimination half-lives in the mild cases were 9.9 ± 7.7 hours (mean ± SD), and the serum fenitrothion concentrations declined below the detectable level in 48 hours. The elimination half-lives relating to two severe cases were 5.3 and 6.7 hours in the alpha phase (under direct hemoperfusion), and 35 and 52 hours in the beta phase, respectively. The serum fenitrothion concentrations fell below the detectable level in 300 hours.

Permethrin: Regarding its metabolism, permethrin is converted to 2,2-dichlorovinyl-2,2-dimethylcyclopropane-1-carboxylic acid and 3-phenoxybenzyl alcohol (3PBAlc) by carboxyl-esterase. The latter metabolite is followed by oxidation to form 3-phenoxybenzaldehyde, and finally 3-phenoxybenzoic acid (3PBA) (Figure 2). The metabolites are reported to be endocrine-disrupting agents, but most studies mention permethrin toxicity is derived from itself (Yuan et al., 2010). In general, *trans* isomer is more rapidly metabolized than *cis* isomer, which is related to the lower susceptibility of *cis* isomer to enzymatic hydrolysis of the ester linkage (Soderlund and Casida, 1977; Zhang et al., 2008). Besides hydrolytic pathway by carboxylesterase, oxidative metabolic pathway of both *cis*-and *trans*-permethrin in rat and human hepatic microsomes was recently reported (Scollon et al., 2009). The toxicokinetics of permethrin (with a *cis*:*trans* ratio of 25:75) was investigated after single oral doses to rats (Anadon et al., 1991). The plasma level of permethrin was maximal within 4 hours after dosing, and then was slowly eliminated from plasma with a half-life of 12.4 hours. The bioavailability of permethrin was found to be 60.7%. The maximum permethrin concentrations in the central and peripheral nervous system were higher than plasma concentrations, and declined with half-life similar to those of plasma. Clearance of *trans*- and *cis*-permethrin from the blood was also investigated in a man who drank an emulsifiable concentrate formulation of permethrin (consisting of 43.5% *cis* and 56.5% *trans*) (Gotoh et al., 1998). The serum concentrations of *cis*- and *trans*-permethrin peaked 3-4 hours after ingestion and then declined, with *trans*-permethrin cleared from the blood more quickly than *cis*-permethrin. Levels of the *trans* isomer were below the detectability threshold within 25 hours after exposure, whereas *cis* isomer was still detectable 10 days after exposure. The present study indicated that the differential persistence of *cis* and

trans isomers in human is consistent with a difference in the metabolic rate of *cis*- and *trans*-permethrin in animal studies (Anadon et al., 1991).

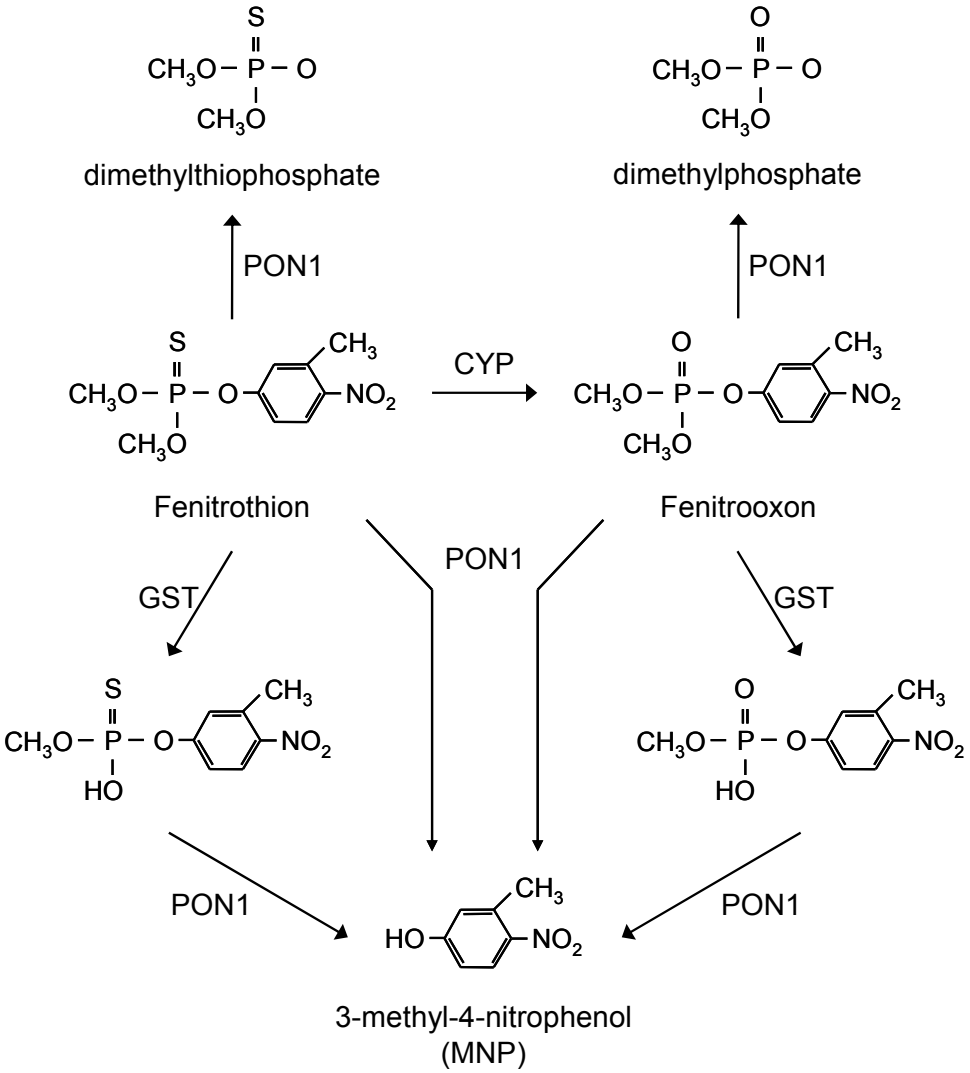


Fig. 1. Metabolic pathway of fenitrothion in mammals or birds. This figure was adopted from WHO (1992) with slight modifications. In mammals, dimethylthiophosphate, dimethylphosphate and MNP were the major urinary metabolites of fenitrothion and fenitrooxon. In birds, MNP was the major urinary metabolite of fenitrothion and fenitrooxon. GST activity was found to be lower than in mammals.

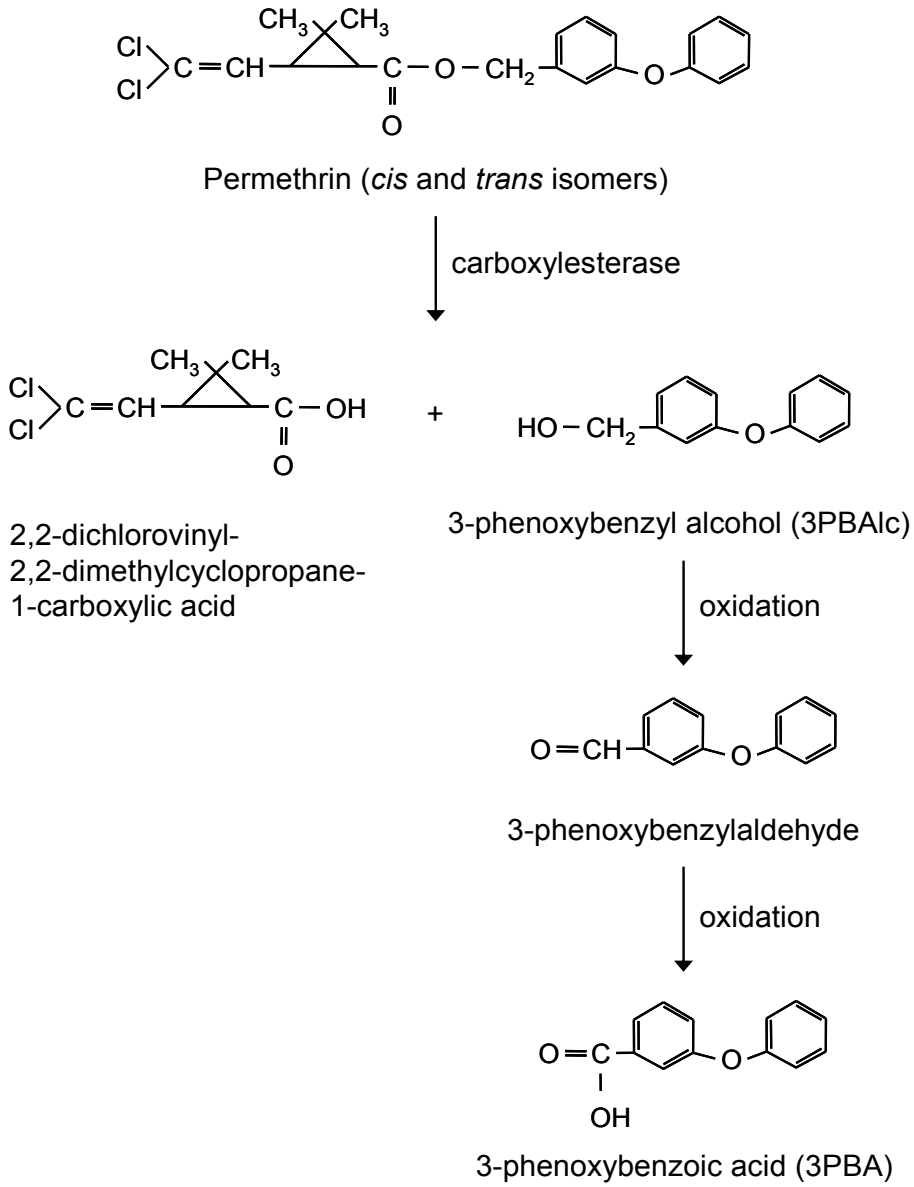


Fig. 2. Metabolic pathway of permethrin in mammals. This figure was adopted from WHO (1990) with slight modifications. Carboxylesterase plays an important role in the hydrolytic biotransformation of permethrin. The expression of carboxylesterase is ubiquitous with high levels in various tissues. Amon various animal tissues, the highest hydrolase activity is typically found in the liver and other tissues, such as testis, kidney and plasma. Unlike mouse, rat, rabbit, horse and cat, human plasma contains no carboxylesterase.

3. Toxicity for experimental animals and humans

Fenitrothion

Since it is a cholinesterase (ChE) inhibitor, exposure to fenitrothion causes ChE activity depression in plasma, red blood cells, brain, and liver tissues. The acute toxicity of fenitrothion is considered to be low in mammals, because of the high metabolic rate (Hayes, 1982; Spencer, 1981).

Animal: The no-observed-adverse-effect levels (NOAEL), based on brain-ChE activity, were 10 mg/kg diet in both short- and long-term studies on rats, in long-term studies on mice, and were 50 mg/kg diet in short-term studies on dogs, respectively (WHO, 1992). Fenitrothion was given to female rats by gavage every other day from gestational day 6-15 at doses 3, 15, 30 and 45 mg/kg (Berlińska and Sitarek 1997). The maternal death rates were 39% and 88% at doses of 30 and 45 mg/kg, respectively. At 30 mg/kg, fenitrothion caused a significant decrease in maternal body weight gain, food consumption, hemoglobin and hematocrit values, and absolute weights of liver and kidney, but an increase in relative weights of adrenal and ovary. At 15 mg/kg, fenitrothion significantly decreased maternal relative liver weight. Although fenitrothion at doses of 3-30 mg/kg did not induced teratogenic effects, at 30 mg/kg it showed embryotoxicity, such as a significant increase in the frequency of early resorption per litter, postimplantation loss, and fetuses and litters with dilation of the cerebral ventricles. Furthermore, fenitrothion produced delayed ossification of sternum and cranium, and decreased fetal body weight and length. The frequency of fetuses and litters with dilation of the cerebral ventricles was increased at a dose of 15 mg/kg. Thus, Berlińska and Sitarek concluded that the NOAEL for developmental toxicity in rats was 3 mg/kg per day, and the lowest-observed-adverse-effect level was 15 mg/kg per day.

Recent studies showed the endocrine-disrupting effect of fenitrothion. Berger and Sultatos (1997) demonstrated that fenitrothion treatment caused a dose-dependent decrease in 2-hydroxyestradiol and 4-hydroxyestradiol production in mouse hepatic microsomes even at a dosage as low as 7 mg/kg, and an increase in 16 alpha-hydroxyestrone and estriol production. In another study, 7-week-old castrated Sprague-Dawley rats were subcutaneously treated with testosterone propionate (50 µg/day in 0.2 ml corn oil) and orally with corn oil vehicle or fenitrothion (15 or 30 mg/kg per day) once a day for 7 days (Tamura et al. 2001). Both fenitrothion doses caused significant decreases in the weights of ventral prostate, seminal vesicle, and levator ani plus bulbocavernosus muscles. In contrast, blood acetylcholinesterase activity was only inhibited at the higher dose (30 mg/kg). Tamura et al. also demonstrated in an *in vitro* experiment that fenitrothion blocked dihydrotestosterone-dependent androgen receptor (AR) activity in a concentration-dependent and competitive manner in HepG2 human hepatoma liver cells, which were transiently transfected with human AR and an AR-dependent luciferase reporter gene, suggesting that fenitrothion may be a competitive androgen receptor antagonist.

On the other hand, Okahashi et al. (2005) suggested that lower-dose fenitrothion did not cause disruption of endocrine systems in animals. They administered fenitrothion to Crj:CD(SD)IGS parental rats at concentrations of 10, 20, and 60 (3.81 mg/kg per day) ppm in the diet for 10 weeks prior to mating, and throughout mating, gestation and lactation. Their offspring were exposed from weaning until maturation at the age of 10 weeks. In the parental animals, brain cholinesterase activity was remarkably reduced in males exposed to 60 ppm fenitrothion and in females exposed to 20 and 60 ppm fenitrothion. Reproductive

performance, organ weights, histopathology, and sperm analytical parameters were not influenced. In the offspring, no effects on anogenital distance, retention of areolae/nipples, onset of puberty, organ weights, histopathological findings, and sperm parameters were observed. In conclusion, fenitrothion had no effects on the reproductive or endocrine systems of the parental animals and their offspring, even at a toxic dose suppressing brain cholinesterase activity in parental animals. The concentration of 60 ppm (3.81 mg/kg per day) is 750 times higher than the acceptable daily intake (ADI) of fenitrothion (0.005 mg/kg body weight). Therefore, any potential risk of exposure may be negligible, and fenitrothion at in-use levels in the environment may be unlikely to cause disruption of human endocrine systems.

Human: WHO (1990) classified technical grade fenitrothion as "moderately hazardous" (Class II). ADI of fenitrothion was established as 0.005 mg/kg body weight by the Joint FAO/WHO Expert Committee on Pesticide Residues in 2000. Nosal and Hladka (1968) reported that administration of fenitrothion at a single oral dose of 0.042-0.33 mg/kg body weight and repeated administration of 0.04-0.08 mg/kg body weight to human volunteers did not cause inhibition in plasma and erythrocyte ChE, and the urinary MNP was completely excreted within 24 hours. Chronic symptoms of exposure to fenitrothion in humans include general malaise, fatigue, headache, loss of memory and ability to concentrate, anorexia, nausea, thirst, loss of weight, cramps, muscular weakness and tremors.

Permethrin

Permethrin acts on the axons in the peripheral and central nervous systems, causing prolonged opening of sodium channels. The acute toxicity of permethrin in mammalians is relatively low, though the LD50 value varies considerably according to the vehicle used and the *cis*:*trans* isomeric ratio (FAO, 1999; U.S. EPA, 2007).

Animal: NOAEL is assigned at 5 mg/kg body weight per day for permethrin with an isomer ratio of *cis*:*trans* 40:60 from the viewpoint of the effects on liver weight in 2-year and 26-week studies in rats, and a 3-month study in dogs. NOAEL is not available for respective *cis* and *trans* isomers (WHO, 1990). The rat appeared to be the most sensitive species with an oral LD50 of 400 mg/kg body weight for *cis*:*trans* 40:60 permethrin administered in corn oil, against 650 mg/kg body weight in mice. The neurotoxicity of intravenous- or orally-administered *cis*-permethrin is over 10-fold greater than that of *trans* isomer. Neonatal rats are more sensitive than adult rats to the acute toxic effects of permethrin, which are thought to be related to differences in permethrin metabolism.

In their acute neurotoxicity study, Freeman (1993a) performed a functional observational battery (FOB) approximately 12 hours following administration of 10, 150 or 300 mg/kg of technical grade permethrin (mixture of *cis* and *trans*) in corn oil to male and female Sprague-Dawley rats. At doses of 150 and 300 mg/kg, permethrin caused salivation, tremor, splayed hindlimbs, abnormal posture, staggered gait, decreased grip strength, exaggerated reaction to sound, exaggerated hindlimb flexion, convulsions, and mortality. No treatment-related effects were observed at the lowest dose of 10 mg/kg. In a behavioral neurotoxicity study (McDaniel and Moser, 1993), technical grade permethrin was administered by gavage in corn oil to Long-Evans hooded rats, at doses of 25, 75 and 150 mg/kg, and the FOB were evaluated at 2 and 4 hours following treatment. Results of the present study are consistent with the acute regulatory study (Freeman, 1993a) including tremor, chromodacryorrhea, decreased grip strength and an exaggerated startle response. However, the absence of salivation, splayed hindlimbs and convulsions and the presence of aggressive sparring in

the latter study (McDaniel and Moser, 1993) were inconsistent with the findings of the regulatory acute neurotoxicity study (Freeman, 1993a).
In a subchronic neurotoxicity study (Freeman, 1993b), technical grade permethrin was administered through the diet to male and female Sprague-Dawley rats, at concentrations of 250, 1500 and 2500 ppm (18, 101 and 170 mg/kg per day, respectively). At the 1500 and 2500 ppm dietary levels, permethrin produced tremor, splayed hindlimbs, abnormal posture, a staggered gait, and decreased grip strength. No such effects were observed at the lowest dose of 250 ppm.
Effects of permethrin on endocrine or reproductive function are investigated, but the report is very limited. Castrated rats (5-week-old) were pretreated with testosterone propionate and orally given permethrin (mixture of *cis* and *trans*, 24.8% and 71.8%) at doses of 10, 50 and 100 mg/kg per day for 10 days. A mixture of *cis*- and *trans*-permethrin showed anti-androgen-like effects on male rats such as significant reductions in androgen-dependent sex accessory tissue (ventral prostate, seminal vesicles, levator ani and bulbocavernosus muscles, Cowper's gland and glans penis) weights (Kim et al., 2005). *cis*-Permethrin at 0, 35 and 70 mg/kg was orally administered to IRC mice for 6 weeks, and male reproductive toxicity was investigated. This chemical dose-dependently decreased testicular and plasma testosterone levels, along with a dose-dependent increase in circulating LH and declines in epididymal sperm count and sperm motility (Zhang et al., 2007). Testicular residue concentrations of *cis*-permethrin from the individual animals were also strongly inversely correlated with testicular testosterone levels. The exposure-related reductions in mRNA and protein expression levels of peripheral benzodiazepine receptor, steroidogenic acute regulatory protein and cytochrome P450 side-chain cleavage, which are involved in testosterone synthesis in testis, were observed, as well as structural changes in Leydig cell mitochondria, suggesting that the mitochondrial damage caused by permethrin exposure may result in a reduction of testosterone synthesizing elements and thereby decrease testosterone levels. In a follow-up study in mice, *cis*-permethrin induces reproductive toxicity whereas at the same dose *trans*-permethrin does not because of a faster metabolic rate than *cis* isomer (Zhang et al., 2008). Zhang et al. also reported that *cis*-permethrin caused structural abnormalities in the seminiferous tubules. However, it must be noted that these studies to date have used dose levels much higher than encountered by non-occupationally exposed humans.
Human: Permethrin is a moderately to practically non-toxic pesticide in EPA toxicity class II or III, depending on the formulation. Formulations in the case of possible eye and skin irritation are grouped into class II. Permethrin belongs to the type I group of pyrethroids because it lacks a cyano group, and typically causes tremor (T-syndrome), incoordination, hyperactivity, prostration, and paralysis. An ADI of 0.05 mg/kg body weight for *cis*:*trans* 40:60 or 25:75 permethrin was established in 1987. Rishikesh et al. (1978) evaluated staff involved with bagging, mixing, or spraying a 5% preparation of permethrin (cis/trans ratio, 25:75) in Nigeria by a questionnaire and urinalysis. Regardless of the protective equipment worn by the sprayers, only 2 mg of permethrin was absorbed after exposure to 6 kg of permethrin, which was excreted in 24 hours.

4. Toxicity for ecosystem

Fenitrothion

In the environment, fenitrothion is degraded by photolysis and hydrolysis. In the presence of ultraviolet radiation or sunlight, the half-life of fenitrothion in water is less than 24 hours.

The presence of micro-flora may accelerate degradation. Miyamoto et al. (1966) studied the degradation of fenitrothion by *B. subtilis*. The major metabolite was aminofenitrothion, and other minor metabolites detected were dimethyl thiophosphoric acid and desmethyl fenitrothion. In the bacteria, aminofenitrothion is further degraded to desmethyl aminofenitrothion, but the rate is slower than the parent compound. No reduction of desmethyl fenitrothion to desmethyl aminofenitrothion was detected, and dimethyl phosphoric acid was not formed from aminofenitrothion (Figure 3). Thus, the degradation of fenitrothine in *B. subtilis*, may be quite different from the metabolic route of experimental animals and humans. In the absence of sunlight or microbial contamination, fenitrothion is stable in water. In soil, biodegradation is the primary route, though photolysis may also play a role. Airborne concentrations of fenitrothion and its levels in water may decrease rapidly by photolysis and hydrolysis. The concentrations of fenitrothion that are likely to be found in the environment do not have any effects on microorganisms in soil or water. In laboratory studies, fenitrothion is highly toxic for aquatic invertebrates in both freshwater and seawater, while fish are less sensitive to fenitrothion than invertebrates, and the most sensitive life stage is early larva. Fenitrothion is highly toxic to bees (LD50, 0.03-0.04 μg/bee) when bees are exposed to direct treatment or to dried residues on foliage (U.S. EPA, 1987).
Furthermore, fenitrothion was found to be highly toxic to upland game birds, but not so toxic to waterfowl. Indeed, the acute oral LD50 values were determined to be 23.6 and 1190 mg/kg body weight for bobwhite quail and mallards, respectively. Even in reproduction studies, NOEL was 10 mg/kg body weight for the quail and 100 mg/kg body weight for the mallard, respectively. There are quantitative differences in the composition of metabolites of fenitrothion between mammalian and avian species. For example, in rats, mice and dogs, demethylated products at the *P*-*O*-methyl linkage by GST accounted for 30 to 60% of the total urinary metabolites (Hollingworth et al., 1967; Miyamoto et al., 1976), whereas in the birds only 10 to 15%. This may be due to lower GST activity in avian species compared with that in mammalians. Mihara et al. (1979) also revealed that oxidative activities of the *m*-methyl group of fenitrothion and fenitrooxon in livers from hen, quail, pheasant and duck were higher than those of mammalian liver, while *O*-demethylate activity for fenitrothion or fenitrooxon was lower in these birds. In birds, MNP is the major metabolite of fenitrothion by hydrolysis, though a pathway exists with oxidation of the *m*-methyl group of fenitrothion or fenitrooxon. The metabolite MNP is then conjugated with uridine diphosphate glucuronic acid or 3'-phosphoadenosine-5'-phosphosulfate by catalytic action of uridine diphosphate glucuronosyltranferase (UGT) and sulfotransferase (SULT), respectively (Mackenzie et al., 1997). Hepatic UGT and SULT activities investigated in vitro for MNP in Japanese quail, mice and rats revealed lower UGT activity for MNP in quail than rats and mice, but no significant difference in SULT activity (Lee et al., 2007). In addition, the SULT activity was only one-tenth of the UGT activity, suggesting that the latter enzyme plays an important role in MNP elimination in vivo. Li et al. (2008) reported that the birds treated with 100 mg/kg of MNP induced acute toxicological responses such as dyspnea and tremor, and finally death. MNP may cause acute toxicity and death, possibly by a rapid decrease in blood pressure followed by ischemic shock, because the potential vasodilatory action of MNP had been reported in rats (Mori et al., 2003; Taneda et al., 2004). However, none of the rats died after treatment with 100 mg/kg of MNP (Li et al., 2007), suggesting that the sensitivity to MNP is higher in quail than in rats. For these reasons, fenitrothion causes higher toxicity in birds than in mammals.

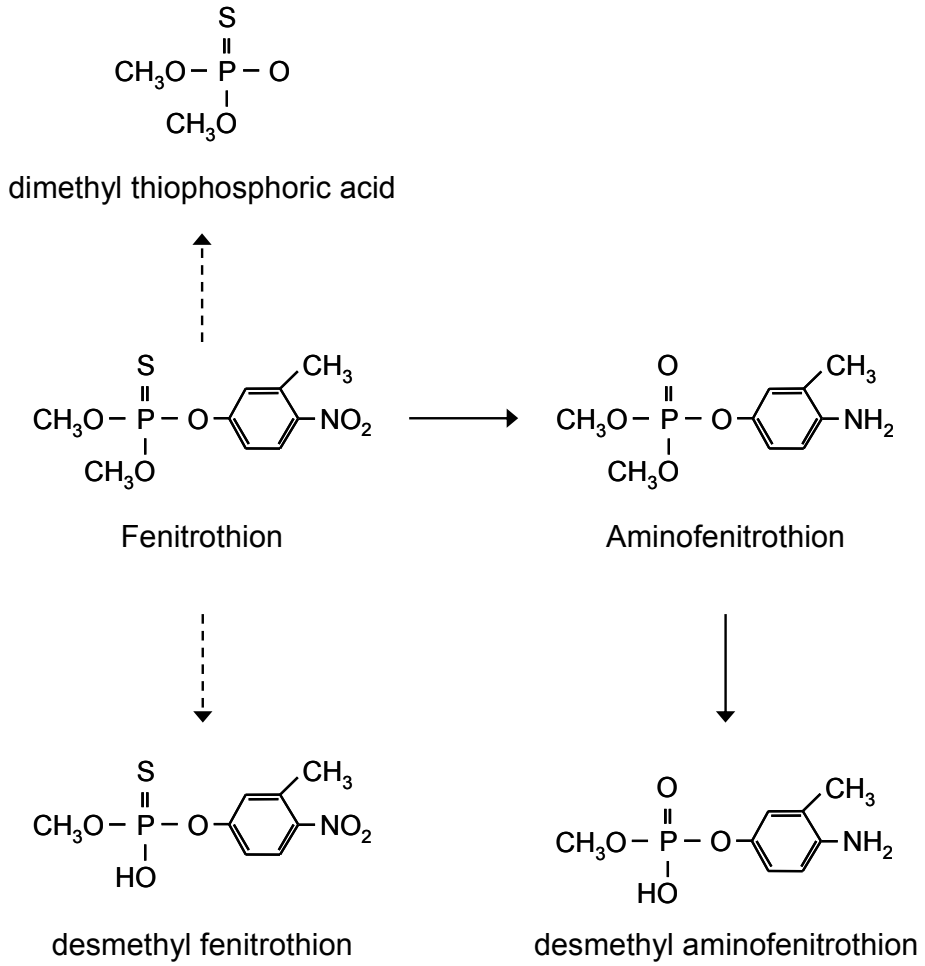


Fig. 3. Pathway of degradation of fenitrothion by *B. subtilis* in environment. This figure was adopted from Miyamoto et al. (1966) with slight modifications. Aminofenitrothion was the major metabolite of fenitrothion in *B. subtilis.*

Permethrin

Permethrin is photodegraded by sunlight in water and on soil surfaces. Under aerobic conditions in soil, permethrin degrades with a half-life of 28 days. Permethrin deposited on plants degrades with a half-life of approximately 10 days. Thus, in the environment, permethrin is hydrolyzed, and the resultant acid and alcohol are conjugated. However, permethrin itself evidences very little movement within the environment, because it binds very strongly to soil particles and is nearly insoluble in water and not expected to leach or contaminate groundwater. Permethrin has been shown to be highly toxic for aquatic arthropods and fish, because they have lower levels of carboxylesterase activity than

mammals. However, the extreme susceptibility to permethrin may be ascribed to its high sensitivity to sodium channels rather than low carboxylesterase activity. Permethrin is also highly toxic to honey bees (LD50, 0.11 μg/bee), yet exhibits very low toxicity to birds when given orally or fed in the diet. LD50 is >3000 mg/kg body weight for a single oral dosage and >5000 mg/kg diet for dietary exposure, respectively. One of the reasons for the different toxicity of permethrin among species is negatively correlated its toxic action to their body temperature, thus generally showing more acute effects on cold-blooded animals (insects, fish, etc) than warm-blooded animals (mammals and birds).

5. Interactive toxicity of insecticide mixture

Organophosphorus insecticides are being increasingly used in combination with pyrethroid insecticides. Fenitrothion is used in combination with other pesticides to enhance ChE inhibition by nature. However, fenitrothion inhibits not only ChE but also other esterase activity such as carboxylesterase. Trottier et al. (1980) reported that the oral administration of fenitrothion to male CD rats at a dose of 0, 2.5, 5, 10, or 20 mg/kg per day for 30 consecutive days significantly decreased liver carboxylesterase activity (by 50-80%) on days 8-30 at doses more than 2.5 mg/kg per day but had returned to control values by day 45 (15 days after termination of treatment) at all doses except 20 mg/kg per day, at which a decrease of 25% was still observed. At this dose, the values had returned to normal by day 87 (57 days after termination of treatment). A significant decrease in renal carboxylesterase activity (by 20-70%) was also observed on days 8-30 at doses over 5 mg/kg per day. Recovery of the activity was rapid, and the values were comparable to those of controls by day 38 (8 days after the end of treatment).

As described at the metabolism of permethrin, carboxylesterase plays an important role in detoxication of permethrin. Ortiz et al. (1995) examined the interactions between a commercial formulation of methyl parathion and a commercially formulated product of permethrin in male rats. When rats were treated with the mixture, 380 mg/kg of methyl parathion reduced the LD50 of permethrin by only 9.0%, whereas when rats received methyl parathion at 464 mg/kg, the LD50 of permethrin was reduced by 37% ($P < 0.001$). Results indicated that methyl parathion modified the acute toxicity of permethrin. Another study examined the effect of organophosphorus insecticide dichlorvos on excretion levels of urinary *cis*-permethrin-derived 3PBA in rats (Hirosawa et al., 2011). After *cis*-permethrin injection (20 mg/kg) via the tail vein of rats pretreated intraperitoneally with dichlorvos (low dose, 0.3 mg/kg; high dose, 1.5 mg/kg), the amounts of urinary 3PBA excretion over 48 hours were decreased to 81.1% and 70.3% of dichlorvos non-treated rats in the low- and high-dose dichlorvos groups, respectively. The plasma concentration of *cis*-permethrin-derived 3PBAlc in high-dose dichlorvos group was significantly lower than that in the dichlorvos non-treated group one hour after *cis*-permethrin injection. In contrast, no differences were observed in the excretion levels of urinary 3PBA after injection of 3PBAlc between the dichlorvos non-treated group and the high-dose dichlorvos group. These results suggested that dichlorvos may have inhibited the metabolism of the co-exposed *cis*-permethrin and thereby decreased the amount of urinary 3PBA excretion. In our recent study, we evaluated male reproductive toxicity after co-exposure to diazinon (3 mg/kg) and *cis*-permethrin (35 mg/kg) in mice. Exposure to diazinon alone and the mixture with *cis*-permethrin inhibited plasma and liver carboxylesterase activities. In the co-exposed mice,

the urinary *cis*-permethrin metabolite decreased compared to that in mice exposed to *cis*-permethrin alone. The co-exposure significantly decreased plasma testosterone levels and increased the number of degenerated germ cells within the seminiferous tubule, whereas exposure to each chemical did not. We concluded that diazinon inhibited the plasma and liver carboxylesterase activities and the metabolic rate of co-exposed *cis*-permethrin, which resulted in accentuating the reproductive toxicity of *cis*-permethrin (Wang et al., unpublished data submitted to the Journal).

Recently, since we could not find any study on the interaction between fenitrothion and permethrin, the toxicity of permethrin may be enhanced by fenitrothion via depression in carboxylesterase activity. Since fenitrothion and permethrin are used in the same place, if not purposefully in mixtures, the two insecticides could conceivably be combined. Until now, the risk assessments of combined toxicity to mammals are still insufficient and further detailed studies are warranted.

6. References

Aardema H, Meertens JH, Ligtenberg JJ: Organophosphorus pesticide poisoning: cases and developments. *Neth J Med* 66:149-153, 2008.

Anadon A, Martinez-Larranaga MR, Diaz MJ, et al: Toxicokinetics of permethrin in the rat. *Toxicol Appl Pharmacol* 110:1-8, 1991.

Berger CW Jr, Sultatos LG: The effects of the phosphorothioate insecticide fenitrothion on mammalian cytochrome P450-dependent metabolism of estradiol. *Fundam Appl Toxicol* 37:150-157, 1997.

Berlińska B, Sitarek K: Disturbances of prenatal development in rats exposed to fenitrothion. *Rocz Panstw Zakl Hig* 48:217-228, 1997.

Clifford NJ, Nies AS: Organophosphate poisoning from wearing a laundered uniform previously contaminated with parathion. *JAMA* 262:3035-3036, 1989.

Elliott M, Farnham AW, Janes NF, et al: A photostable pyrethroid. *Nature* 246:169-170, 1973

Etzel RA, Forthal DN, Hill RH Jr, et al: Fatal parathion poisoning in Sierra Leone. *Bull World Health Organ* 65:645-649, 1987.

Eyer F, Meischner V, Kiderlen D, et al: Human parathion poisoning. A toxicokinetic analysis. *Toxicol Rev* 22:143-163, 2003.

FAO: Pesticide Residues in Food, Toxicological Evaluations; Food and Agriculture Organization of the United Nations and World Health Organization: Rome, 1999.

Freeman C: Permethrin technical: acute neurotoxicity screen in rats. *FMC Corporation* Study No. A92-3646, 1993a.

Freeman C: Permethrin technical: subchronic neurotoxicity screen in rats. *FMC Corporation* Study No. A92-3647, 1993b.

Gotoh Y, Kawakami M, Matsumoto N, et al: Permethrin emulsion ingestion: clinical manifestations and clearance of isomers. *J Toxicol Clin Toxicol* 36:57-61, 1998.

Hayes WJ: Pesticides Studied in Man. *Williams and Wilkins.* Baltimore, London. 1982.

Hayes WJ, Laws ER: Handbook of Pesticide Toxicology, Volume 2: Classes of Pesticides. *Academic Press Inc. NY*, 1990.

Hirosawa N, Ueyama J, Kondo T, et al: Effect of DDVP on urinary excretion levels of pyrethroid metabolite 3-phenoxybenzoic acid in rats. *Toxicol Lett* 203:28-32, 2011.

Hoilingworth RM, Metcalf RL, Fukuto TR: The selectivity of sumithion compared with methylparathion: Metabolism in the white mouse. *J Agric Food Chem* 15:242-249, 1967.

Hoffmann U, Papendorf T: Organophosphate poisonings with parathion and dimethoate. *Intensive Care Med* 32:464-468, 2006.

Kim SS, Lee RD, Lim KJ, et al: Potential estrogenic and antiandrogenic effects of permethrin in rats. *J Reprod Dev* 51:201-210, 2005.

Koyama K, Suzuki R, Kikuno T: Serum fenitrothion concentration and toxic symptom in acute intoxication patients. *Chudoku Kenkyu* 19:41-47, 2006.

Laynez BF, Martínez GL, Tortosa FI, et al: Fatal food poisoning by parathion. *Med Clin (Barc)* 108:224-225, 1997.

Lee CH, Kamijima M, Li C, et al: 3-Methyl-4-nitrophenol metabolism by uridine diphosphate glucuronosyltransferase and sulfotransferase in liver microsomes of mice, rats, and Japanese quail (Coturnix japonica). *Environ Toxicol Chem* 26:1873-1878, 2007.

Li C, Suzuki AK, Takahashi S, et al: Effects of 3-methyl-4-nitrophenol on the reproductive toxicity in female Japanese quail (Coturnix japonica). *Biol Pharm Bull* 31:2158-2161, 2008.

Li C, Taneda S, Suzuki AK, et al: Effects of 3-methyl-4-nitrophenol on the suppression of adrenocortical function in immature male rats. *Biol Pharm Bull* 30:2376-2380, 2007.

Mackenzie PI, Owens IS, Burchell B, et al: The UDP glycosyltransferase gene superfamily: recommended nomenclature update based on evolutionary divergence. *Pharmacogenetics* 7:255-269, 1997.

McDaniel KL, Moser VC: Utility of a neurobehavioral screening battery for differentiating the effects of two pyrethroids, permethrin and cypermethrin. *Neurotoxicol Teratol* 15:71-83, 1993.

Meaklim J, Yang J, Drummer OH: Fenitrothion: toxicokinetics and toxicologic evaluation in human volunteers. *Environ Health Perspect* 111:305-308, 2003.

Mihara K, Misaki Y, Miyamoto J: Metabolism of fenitrothion in birds. *J Pesticide Sci* 4:175-185, 1979.

Miyamoto J, Kitagawa K, Sato Y: Metabolism of organophosphorus insecticides by *Bacillus subtilis*, with special emphasis on Sumithion. *Jpn J Exp Med* 36:211-225, 1966.

Miyamoto J: Mechanism of low toxicity of Sumithion toward mammals. *Residue Rev* 25:251-264, 1969.

Miyamoto J, Mihara K, Hosokawa S: Comparative metabolism of m-methyl-^{14}C-sumithion in several species of mammals *in vivo*. *J Pest Sci* 1:9-21, 1976.

Miyamoto J, Sato Y, Kadota T, et al: Studies on the mode of action of organophosphorus compounds. Part I. Metabolic fate of 32P-labelled Sumithion and methylparathion in guinea-pigs and white rats. *Agric biol Chem* 27:381-389, 1963.

Miyamoto J: Studies on the mode of action of organophosphorus compounds. Part III. Activation and degradation of Sumithion and methylparathion in vivo. Agric biol Chem 28:411-421, 1964.

Mori Y, Kamata K, Toda N, et al: Isolation of nitrophenols from diesel exhaust particles (DEP) as vasodilatation compounds. *Biol Pharm Bull* 26:394-395, 2003.

Nosál M, Hladká A: Determination of the exposure to fenitrothion (*O,O*-dimethyl-O-3-methyl-4-nitrophenyl-thiophosphate) on the basis of the excretion of p-nitro-m-cresol by the urine of the persons tested. *Int Arch Arbeitsmed* 25:28-38, 1968.

Okahashi N, Sano M, Miyata K, et al: Lack of evidence for endocrine disrupting effects in rats exposed to fenitrothion in utero and from weaning to maturation. *Toxicology* 206:17-31, 2005.

Ortiz D, Yáñez L, Gómez H, et al: Acute toxicological effects in rats treated with a mixture of commercially formulated products containing methyl parathion and permethrin. *Ecotoxicol Environ Saf* 32:154-158, 1995.

Rishikesh N, Clarke JL, Martin HL, et al: Evaluation of Decamethrins and Permethrin Against *Anopheles gambiae* and *Anopheles funestus* in a Village Trial in Nigeria. World Health Organization, Division of Vector Biological Control; 1978.

Scollon EJ, Starr JM, Godin SJ, et al: In vitro metabolism of pyrethroid pesticides by rat and human hepatic microsomes and cytochrome P450 isoforms. *Drug Metab Dispos* 37:221-228, 2009.

Soderlund DM, Casida JE: Effects of pyrethroid structure on rates of hydrolysis and oxidation by mouse liver microsomal enzymes. *Pestic Biochem Physiol* 7:391-401, 1977.

Spencer EY: Guide to the Chemicals Used in Crop Protection. 7th edition. Publication 1093. *Research Branch. Agriculture Canada.* 1981.

Tamura H, Maness SC, Reischmann K, et al: Androgen receptor antagonism by the organophosphate insecticide fenitrothion. *Toxicol Sci* 60:56-62, 2001.

Taneda S, Kamata K, Hayashi H, et al: Investigation of vasodilatory substances in diesel exhaust particles (DEP): isolation and identification of nitrophenol derivatives. *J Health Sci* 50:133-141, 2004.

Trottier B, Fraser AR, Planet G, et al: Subacute toxicity of technical fenitrothion in male rats. *Toxicology* 17:29-38, 1980.

U.S. Environmental Protection Agency: Guidance for the Reregistration of Pesticide Products Containing Fenitrothion. US EPA, Office of Pesticide Programs, Registration Div., Washington, DC. 132 pp. July, 1987.

U.S. Environmental Protection Agency: Reregistration Eligibility Decision (RED) for Permethrin. US EPA, Office of Prevention, Pesticides and Toxic Substance, Office of Pesticide Programs, U.S. Government Printing Office: Washington, DC, 2007.

WHO: Environmental Health Criteria 133: Fenitrothion. World Health Organization, International Programme on Chemical Safety, Geneva, Switzerland, 1992.

WHO: Environmental Health Criteria 94: Permethrin. World Health Organization, International Programme on Chemical Safety, Geneva, Switzerland, 1990.

WHO: The WHO recommended classification of pesticides by hazard and Guidelines to classification 1990-1991, Geneva, World Health Organization (WHO/PCS/90.1), 1990.

Worthing CR, Walker SB: The Pesticide Manual: A World Compendium. 8th edition. Published by The British Crop Protection Council, 1987.

Yuan C, Wang C, Gao SQ, et al: Effects of permethrin, cypermethrin and 3-phenoxybenzoic acid on rat sperm motility in vitro evaluated with computer-assisted sperm analysis. *Toxicol In Vitro* 24:382-386, 2010.

Zhang SY, Ito Y, Yamanoshita O, et al: Permethrin may disrupt testosterone biosynthesis via mitochondrial membrane damage of Leydig cells in adult male mouse. *Endocrinology* 148:3941-3949, 2007.

Zhang SY, Ueyama J, Ito Y, et al: Permethrin may induce adult male mouse reproductive toxicity due to *cis* isomer not *trans* isomer. *Toxicology* 248:136-141, 2008.

5

DDT and Its Metabolites in Mexico

Iván Nelinho Pérez Maldonado, Jorge Alejandro Alegría-Torres,
Octavio Gaspar-Ramírez, Francisco Javier Pérez Vázquez,
Sandra Teresa Orta-Garcia and Lucia Guadalupe Pruneda Álvarez
Laboratorio de Toxicología, Facultad de Medicina,
Universidad Autónoma de San Luis Potosí,
San Luis Potosí, SLP,
México

1. Introduction

DDT (dichlorodiphenyltrichloroethane) was first synthesized in 1874, and its insecticidal properties were discovered in 1939 by Paul Hermann Müller (Stapleton 1998). The U.S. military began using DDT extensively for mosquito control in 1944, particularly in the Pacific, where much of the action of World War II took place in highly malarious areas (Stapleton 1998). In 1955, the World Health Organization (WHO) started a global malaria control program with DDT; by 1958, 75 countries had joined and, at the peak of the campaign, 69,500 tons of pesticides mainly DDT [1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane] were applied to 100 million dwellings each year (Wijeyaratne, 1993). For the control of malaria, houses were sprayed twice a year with DDT wettable powder to kill resting mature Anopheles mosquito. Later, the Stockholm Convention on Persistent Organic Pollutants, which came into force on 17 May 2004, outlawed the use of 12 chemicals including DDT (UNEP, 2004). However, one exemption clause allows malaria-endemic nations to use DDT, strictly for disease vector control. The United Nations Environment Program estimates that about 25 countries will use DDT under exemptions from the DDT pesticide ban (POPs, 2009). Thus, in regard to presence of DDT around the world can be divided into three scenarios: Sites where DDT is still in use; sites where the presence is due to DDT sprayed several years ago, and sites where the presence of DDT is the result of a long-range transport of the insecticide to areas where it was never used like the Antarctic. In Mesoamerica (Mexico, Costa Rica, El Salvador, Guatemala, Honduras, Mexico, Nicaragua and Panama) DDT was used until the year 2000, Mexico and Nicaragua being the last nations that applied the insecticide in agriculture and for the control of malaria. Table 1 lists the period and the total amount of DDT used in each Mesoamerican country by the malaria control programs. The amount used (approximately 85,000 tons between 1946 and 1999) together with the high environmental persistence of DDT and its metabolites, provide the necessary conditions for DDT to become a contaminant of concern for this region of the world (ISAT, 2002). Taking into account the environmental persistence and the toxicity of DDT, a program for the control of malaria without using insecticides in Mesoamerica was developed between 2004 and 2007, with assistance from the Pan American Health Organization [PAHO; (Chanon et al., 2003; PAHO, 2008)]. The phase-out of DDT in Costa

Rica, El Salvador, Guatemala, Honduras, Mexico, Nicaragua and Panama was part of a regional proposal supported by the Global Environment Facility (GEF) and the United Nations Environmental Program with the participation of the North American Commission for Environmental Cooperation (CEC).

2. DDT in Mexico

In 1944, and for the first time, houses were sprayed with DDT in Temixco, Morelos, Mexico (Stapleton 1998). The spray was applied to the walls and ceilings of residences. Studies done two months after the spraying, showed that there was a 99% reduction in the incidence of Anopheles (Stapleton 1998). In 1947–48, the spraying of DDT began in other Mexican regions, such as Veracruz, Mexico City and Baja California (Stapleton 1998). By 1948, the first clear evidence of malaria control appeared in the areas first sprayed with DDT; the overall parasite rate in the state of Morelos was found to be 10%, and the rate in the sprayed towns was found to be 1% (Stapleton 1998). In 1936 it was estimated that half of the Mexican population lived in endemic regions and was subject to a malaria mortality rate of 0.5%, or about 36,000 deaths per year (Stapleton 1998).During the 1930s and 1940s, malaria became the third cause of death in the country. However, the antimalaria campaign was not generalized until 1956 (CCE 1998). The success of DDT was outstanding, malaria cases decreased from 41,000 in 1955 to 4,000 in 1960 (Fernández de Castro 1998); in 1970 the campaign was relaxed and the cases increased to 57,000 (Fernández de Castro 1998). However, this was also the time in which DDT production peaked in Mexico, with more than 80 thousand tonnes produced annually (CCE 1998). In recent years, the incidences of malaria have declined significantly, to less than 5,000 cases. Since 1982 there have been no deaths from this disease. As, showed, Malaria is a long-standing public health problem that has inhibited development in large areas of the country. Approximately 60% of the Mexican territory, representing an area inhabited by close to 45 million people, provides an environment suitable for malaria transmission. This includes the Pacific coast, the Gulf of Mexico slopes, the Yucatan peninsula and interior basins of the high plateau. (CCE 1998). In actuality, Mexico operates a malaria control program that has substantially reduced the incidence of this disease. In 1995, Mexico initiated an integrated pest management approach for malaria to reduce the heavy dependence on pesticides. Much of the success of Mexico's malaria control program (there have been no recorded deaths from malaria since 1982) is due to improvements in sanitation, increased disease surveillance, and integrated pest management schemes that focus pesticide applications on critical habitats and stages in the mosquito's life cycle (Government of Mexico 1998). Since 1998, DDT was substituted with pyrethroids in the malaria control program. In other hand, In the area of agriculture, as much as 1,000 tonnes per year were used (CCE 1998).Application rates in the north of Mexico, were among the highest in the world (CCE 1998). However, the growing concern about DDT persistence has had a significant impact on agricultural practices in Mexico. During the early 1970s the US Food and Drug Administration (USFDA) began rejecting the importation of commodities due to high residue levels, especially of DDT (CCE 1998). Therefore, some agricultural areas changed to newer pesticides in order to comply with the USFDA regulations. By 1990, DDT was limited to campaigns addressing public sanitation (CCE 1998). In recognition of DDT's environmental and human health effects, Mexico shifted the emphasis of its anti-malarial campaigns away from DDT beginning in

the 1980s and 1990s, and the use of the pesticide was gradually reduced. In 1997, the Intergovernmental Forum on Chemical Safety agreed there was sufficient evidence to take international action to restrict and reduce the use of DDT.

Country	Period of use	Total tons
México	1957-2000	69,545.00
Nicaragua	1959-1991	2,172.00
Costa Rica	1957-1985	1,387.00
Guatemala	1958-1979	4,790.00
Honduras	1950-1978	2,640.00
El Salvador	1946-1973	4,271.00
Panamá	1967-1971	189.00

Table 1. History of DDT use in Mesoamerica countries (ISAT, 2002).

3. Environmental pathways of exposure to DDT

The physicochemical properties of DDTs (Table 2) show the extent of their volatility and the high KOW/KOA value shows that they are more likely to partition into environmental sectors which exhibit greater organic phases (biota, soil, and sediment). The concentration of DDTs in the water samples may be limited due to characteristically low water solubility. In other hand, the exposure pathways are the processes by which DDT may be transported from the pollution source to living organisms. In the malaria areas, the source of DDT was the household-spraying of the insecticide. Since the beginning of the control program of malaria, DDT was sprayed on the ceilings and walls, both indoors and outdoors. Therefore, after spraying, indoor dust (or indoor soil in some cases), and the external surface soil in those areas next to the dwellings, were the media first to become contaminated with DDT. From these points, the insecticide could be transported from one medium to another by different processes.

Compound	Molecular weight	Vapor pressure (Pa)	Aqueous solubility (mol/m^3)	Henry´s law constant (Pa m^3/mol)	Log Kow*	Log Koa
p´p-DDT	354.5	0.00048	0.00042	1.1	6.39	9.73
p´p-DDE	319.0	0.00340	0.00079	4.2	6.93	9.70
p´p-DDD	321.0	0.00120	0.00230	0.5	6.33	10.03

Table 2. Physicochemical properties of DDT and its metabolites at 25^0 C. (Sahsuvar et al. 2003; Shen and Wania 2005).

Soil and Dust

Several studies have identified indoor house dust as an important pathway of toxicant exposure. Often levels of pollutants found in house dust, including compounds banned long ago such as DDT, are significant sources of exposure for the general population, especially children (Butte and Heinzow 2002; Hwang et al. 2008; Rudel et al. 2003). Moreover, analyses of compounds in house dust are a measure of indoor contamination, but may also provide valuable information on the assessment of human indoor exposure (Butte and Heinzow 2002). Also, outdoor soil is considered an important exposure pathway for the general population and children to compounds banned long ago (Herrera-Portugal et al. 2005). However, it is important to note that longer residence times and elevated contaminant concentrations in the indoor environment may increase the chance of exposure to these contaminants by 1,000-fold compared to outdoor exposure (Hwang et al. 2008).
Tables 3 and 4 show DDT levels in outdoor and indoor surface soils, respectively. Taking into account the guideline for DDT in residential soil: 0.7 mg/kg from Canada (Environment Canada, 2007) different scenarios have been observed in Mexico. Regarding outdoor levels, in general lower levels were found in household outdoor samples (Table 3). With exception of levels found in Chiapas and Oaxaca, that have levels lower than Canadian guide (Table 3). In other hand, high levels are recorded in indoor levels in different regions of Mexico, generally higher than Canadian guideline (Table 4). Also, we can note that the higher levels of DDT in those environment media were found in indoor dust samples, generally with levels above the Canadian guideline (Table 5). Moreover, the data in Tables 3, 4 and 5 indicate high levels of total DDT in soil and dust in all regions studied in Mexico when compared with studies around the world.

Water

DDT, DDD and DDE (DDTs) are only slightly soluble in water, with solubilities of 3.4 ppb, 160 ppb and 120 ppb, respectively (ATSDR 2010). In this regard, sedimentation is the most important factor for the disappearance of DDT from water. However, it has also been suggested that contaminated sediments are a main source of DDT inputs to the water column (Zeng 1999). In order to study the degree of pollution in water bodies

located in tropical areas, DDTs were quantified in a relatively small stream. The levels of total DDT found in the tropical area was 280 pg/L (Carvalho et al. 2009). In other hand, levels of total DDT found in the tropical area in United states of America was 10300 pg/L (California 1999)

Sediments

As stated above, sediments act as the primary reservoir for excess quantities of DDT. Therefore, it is very important to analyze the concentrations in this medium. In Table 6 it is shown that DDT concentrations in Mexican samples are lower than those detected in other countries, where DDT was used either for the control of malaria or for agricultural practices. Whether this difference can be explained by an increased degradation or by a DDT mass reduction caused by water currents carrying suspended DDTs out of the contaminated area, are issues that deserve further research. However, we cannot exclude another explanation. The Mexican studies, results of which are shown in Table 6, were not designed to assess the amount of DDT in sediments due to spraying. In fact, a sediment sample collected in a river near an area where the insecticide was used intensively for vector control, had DDT concentrations of up to 70.0 mg/kg (Gonzalez-Mille et al. 2010). Discvutir disminución.

Location	Total DDT (mg/Kg)	Region	Reference
Chiapas	0.95	Southeastern	Martínez-Salinas et al. 2011
Chiapas	8.20	Southeastern	Yañez et al. 2002
Oaxaca	0.90	Southeastern	Yañez et al. 2002
Tabasco	0.04	Southeastern	Torres-Dosal et al. 2011
Chihuahua	0.45	North	Díaz-Barriga et al. 2011
Veracruz	0.01	Southeastern	Espinosa-Reyes et al. 2010
Puebla and Mexico	0.07	Central	Waliszewski et al. 2008

Table 3. Total DDT levels in outdoor surface soil (mg/Kg) in different Mexican Regions.

Mexican state	Total DDT (mg/Kg)	Region	Reference
Chiapas	6.8	Southeastern	Martínez-Salinas et al. 2011
Chiapas	7.1	Southeastern	Yañez et al. 2002
Oaxaca	0.15	Southeastern	Yañez et al. 2002
Chihuahua	0.95	North	Díaz-Barriga et al. 2011

Table 4. Total DDT levels in indoor soil (mg/Kg) in different Mexican Regions.

Food and Biota

Due to their lipophilic attributes and high persistence, the DDTs may bioaccumulate significantly in animal species (Fisher 1995). Furthermore, biomagnification has been observed; for example, DDT concentration increased with each successive trophic level in a food chain (Fisher 1995).Taking into account these properties, food ingestion can be considered a pathway of exposure. In Mexico, studies have been done in different food items, such as fish, hen's egg, butter and cow's milk and muscle. In Table 7, total DDT levels in different food items are presented. Considering fish, the concentrations of DDT, in organisms collected in Mexico, are above normal values. As is shown in Table 8, where DDTs levels in Fish are depicted for different countries. We can note that the food item with high levels of DDT are food rich in fat as butter and cow´s milk (Table 7).

Location	Total DDT (mg/Kg)	Region	Reference
Chiapas	6.9	Southeastern	Martínez-Salinas et al. 2011
Chihuahua	1.0	North	Díaz-Barriga et al. 2011

Table 5. Total DDT levels in dust (mg/Kg) in different Mexican Regions.

Location	Total DDT (μg/Kg)	Region	Reference
Estado, Mexico (bay)	1.5	Southeastern	Noreña-Barroso et al. 1998
Mexico (bay)	0.6	Southeastern	Noreña-Barroso et al. 2007
Mexico (river)	74.0	Southeastern	Gonzalez-Mille et al. 2010
Mexico (lagoon)	4.9	Southeastern	Botello et al. 2000
China (Bay)	7.8		Liu et al. 2011
China (river)	3.8		Tan et al. 2009
Korea (bay)	3.4		Khim et al. 2001
Japan (bay)	1.2		Kim et al. 2007

Table 6. Total DDT levels in sediment (mg/Kg) in different Mexican Regions.

Air

Because DDTs have a Henry's Law constant value of 10–4/10–5 atm m3 mol, they are considered moderate volatile compounds [5]. Therefore, these compounds can be transported by air, either in the gaseous phase or adsorbed to atmospheric particles [5].Photodegradation of DDT occurs slowly; thus, residues of these pesticides are ubiquitous in the atmosphere, although at lower concentrations. Information on the atmospheric levels of OCs in Mexico is scarce. Previous studies in southern Mexico found that DDT and toxaphene concentrations in air were 1-2 orders of magnitude above levels in the Laurentian Great Lakes and arctic regions (24-26). Atmospheric levels in southern Mexico were generally higher than those in central Mexico (27), Costa Rica (28, 29) and Cuba (30), and comparable to those in Belize (31).

Recently, two important studies regarding DDT and its metabolites in air has been developed. Passive air samplers (PAS) were deployed at four sampling sites at the southern Mexico in 2002-2004 and eleven sampling sites across Mexico during 2005-2006 (referencia). The total DDT levels ranged from 239 to 2360 pg/m^3 in 2002-2004 (referencia) and from 15 to 1975 pg/m^3 in 2005-2006 (refernecia). Table ? shows the Total DDT air levels in the sampling sites in both studies. Total DDT tended to be higher in the south (poner siglas) and

some central sites (sigles). While, the other central and northern sites had lower total DDT levels (poner siglas). It is important to note that the higher levels were found in tropical sites where DDT was used for health campagnes or for agriculture as MT, CEL and VC (Table ?).

Location	Food	Total DDT (mg/Kg)	Region	Reference
Mexico country	Butter	88.0		Waliszewski et al. 2003
Veracruz	Cow´s milk	39.0	Southeastern	Pardio et al. 2003
Campeche	Oysters	5.9	Southeastern	Carvalho et al. 2009
Campeche	Oysters	1.5	Southeastern	Gold-Bouchot et al. 1995
Tabasco	Oysters	6.2	Southeastern	Botello et al. 1994
Campeche	Shrimps	0.25	Southeastern	Gold-Bouchot et al. 1995
Campeche	Mussels	1.44	Southeastern	Gold-Bouchot et al. 1995
Baja California	Mussels	9.16	North	Gutierrez-Galindo et al. 1988

Table 7. Total DDT levels in food items (mg/Kg) in different Mexican Regions.

Location	Total DDT (ng/g lipid)	Region	Reference
Veracruz, Mexico	25.0	Southeastern	Gonzalez-Mille et al. 2010
Chiapas, Mexico	4.7	Southeastern	Pérez-Maldonado et al. 2010
Hidalgo, Mexico	7.3	Central	Fernandez-Bringas et al. 2008
Costa Rica	0.6		Pérez-Maldonado et al. 2010
Honduras	< LOD (below detection limit)		Pérez-Maldonado et al. 2010
Nicaragua	3.9		Pérez-Maldonado et al. 2010
El Salvador	3.8		Pérez-Maldonado et al. 2010
Guatemala	< LOD		Pérez-Maldonado et al. 2010

Table 8. Total DDT levels in fish (mg/Kg) in different Mexican Regions.

Community	Total DDT (pg/m3)	Region	Reference
Baja California	338	North	Wong et al. 2009
Chihuahua	34	North	Wong et al. 2009
Yucatan	1975	Southeastern	Wong et al. 2009
Colima	750	North	Wong et al. 2009
Veracruz	129	Southeastern	Wong et al. 2009

Community	Total DDT (pg/m3)	Region	Reference
Morelos	500	Central	Wong et al. 2009
Sinaloa	76	North	Wong et al. 2009
Mexico DF	55	Central	Wong et al. 2009
Nuevo Leon	15	North	Wong et al. 2009
San Luis Potosi	21	Central	Wong et al. 2009
Veracruz	50	Southeastern	Wong et al. 2009
Tabasco	239	Southeastern	Alegría et al. 2008
Chiapas	2360	Southeastern	Alegría et al. 2008
Chiapas	547	Southeastern	Alegría et al. 2008
Veracruz	1200	Southeastern	Alegría et al. 2008

Table 9. Total DDT levels in air (pg/m3) in different Mexican Regions.

4. Human exposure to DDT

Biomonitoring studies are a useful instrument in formulating environmental health policies. For example, in Mexico during the last decade, studies in children led to the reduction or elimination of different chemicals such as lead, lindane and inclusive DDT. Furthermore, the biomonitoring of susceptible populations is a valuable method for the identification of critical contaminants, as has been shown in the United States with the National Health and Nutritional Examination Survey (NHANES III; Needham et al., 2005). Information about human exposure to chemicals is very limited; besides, in relation to children, the information is even more scarce. In this regard, in this text, we shall present data for breast milk, blood serum and adipose tisssue. Results have been done in adults and children.

In other hand, a biomarker of exposure is a xenobiotic substance or its metabolite (s), that is measured within a compartment of an organism. The preferred biomarkers of exposure are generally the substance itself or substance-specific metabolites in readily obtainable body fluid(s) or excreta. DDT and its metabolites DDD,DDE, DDA, and MeSO2-DDE (3-methylsulphonyl-DDE), can be measured in adipose tissue, blood serum, urine, feces, semen, or breast milk .

Breast Milk

Psychological and medical studies have underlined the benefits of nursing which raises immunological defenses and provides a healthier development of the baby. Parallel findings have increased concern about the excretion of drugs and environmental contaminants contained in breast milk, since it is considered the main route for eliminating deposited organochlorine pesticides from a mother's body (Jensen and Slorach 1991; Sonawane 1995; Cupul-Uicab et al. 2008).

Because of their lipophilic nature and high persistence, DDT and its metabolites accumulate in lipophilic human body parts, particularly in lipid-rich tissues such as adipose tissue and subsequently translocated and excreted through milk fat. A major concern is that milk is the first (and in some areas the only) food for the newborn child.

Concentrations of DDTs (DDT, DDD and DDE) in human milk have been shown to be higher in communities exposed to this insecticide, than in non-exposed populations (Table 10). For example, levels from a cotton area where DDT was used for agricultural purposes (Coahuila) were higher or similar to those obtained in samples collected in a malarious area (Veracruz, Yucatan) where DDT was extensively used (Table 10). And both were higher than the concentrations quantified in urban areas (Mexico DF), where DDT has never been used.

The World Health Organization's Acceptable Daily Intake (ADI) for DDTs is 20 mg/kg/day (Lu 1995). In this regard, considering a body mass of 5 kg, a milk intake of 0.85 kg/day, a proportion of fat in milk of 0.035, and a DDT concentration in milk of 10.4 mg/kg (total DDT concentration in samples collected during 1996 1997 in a suburban malarious area; Albert et al. 1980), the estimated daily intake is three times higher than the ADI. Furthermore, if the maximum range concentration found in some studies (36.5 mg/kg; Albert et al. 1980) is taken into account, the ADI is surpassed 11 times. However, when calculated the ADI with DDT concentration in milk of 2.4 mg/kg (Waliszewski et al. 2009), the ADI calculated is lower than 20 mg/kg/day. It is important to remark that the chronological levels obtained by Waliszewski et al. (1996, 1999, 2001, 2002, 2009) have a decreasing tendency. That result coincides with the restriction and prohibition for DDT use in Mexico.

Serum

In this document, we presented data regarding DDT and its metabolites levels in children (Table 11) and adults (Table 12)

Children appear to be particularly suitable for a monitoring program, as they are not directly exposed to occupational pollution; thus, children normally reflect present trends of environmental exposure more accurately than do adults (Link et al., 2005). Moreover, it is well established that children are potentially at a higher risk than adults for adverse health effects from exposure to many environmental chemicals (Guzelian et al., 1992; Bearer, 1995; Carlson, 1998; Galson et al., 1998; Aprea et al., 2000; Needham and Sexton, 2000; Adgate and Sexton, 2001; Brent et al., 2004; IPCS, 2006).

Location	Total DDT	Region	Reference
Coahuila	10400.0	North	Albert et al. 1980
Mexico DF	900.0	Central	Torres-Arreola et al. 1999
Veracruz	7815.0	Southeastern	Pardio et al. 1998
Veracruz	6280.0	Southeastern	Waliszewski et al. 1996
Veracruz	4700.0	Southeastern	Waliszewski et al. 1999
Veracruz	4700.0	Southeastern	Waliszewski et al. 2001
Veracruz	3740.0	Southeastern	Waliszewski et al. 2002
Morelos	4320.0	Central	Lara et al. 2000
Yucatan	3065.0	Southeastern	Rodas-.Ortíz et al. 2008
Veracruz	2335.0	Southeastern	Waliszewski et al. 2009

Table 10. Total DDT levels in human milk (ng/g lipid) in different Mexican Regions.

Adipose tissue

Adipose tissue biopsy has been used in epidemiological studies to assess chronic exposure to DDT. This is a logical choice because the DDTs are accumulated in adipose tissue due to its lipid solubility. The half-life of DDT in human adipose tissue is approximately seven years (Woodruff et al. 1994).

As in serum, DDTs in adipose tissue are a good biomarker of exposure for communities exposed to DDT. When compared to an urban non-exposed community [46], the levels of DDTs (especially those of DDE), were higher in the exposed population (Table 9). In the same table it can be observed that the concentrations of DDT in adipose tissue from workers of the malaria program were higher than the levels found in people living in an agricultural area or in malarious areas. In the workers, a linear model that included an index of chronic exposure, the use of protective gear,and recent weight loss explained 55% of the variation of

p,p′-DDE conncentrations in adipose tissue. The index of chronic exposure was constructed according to worker position and based on the historical duration and intensity of DDT application [48].
When the concentrations of DDTs in adipose tissue were expressed by age group, two groups were identified as the most exposed. Those groups were children and elderly people [49]. The levels in elderly people can be explained by the accumulation of DDT in a chronic exposure scenario,whereas the concentration in children may be the result of an exposure to multiple pathways (soil, household dust, air, water, food, etc.). It is interesting that the group less exposed to DDT was the 0–2 years, a group that may be exposed to DDT through lactation [49].

Location	Total DDT (ng/g lipid)	Region	Reference
Chiapas	22280.0	Southeastern	Trejo-Acevedo et al. 2009
Oaxaca	7500.00	Southeastern	Perez-Maldonado et al. 2006
Quintana Roo	11300.0	Southeastern	Perez-Maldonado et al. 2006
Chihuahua	35000.00	North	Díaz-Barriga et al. 2011
Queretaro	2170.0	Central	Trejo-Acevedo et al. 2009
Durango	2270.0	North	Trejo-Acevedo et al. 2009
San Luis Potosí	1990.0	Central	Trejo-Acevedo et al. 2009
Guanajuato	940.0	Central	Trejo-Acevedo et al. 2009
Veracruz	1910.0	Southeastern	Trejo-Acevedo et al. 2009
Michoacan	550.0	Central	Trejo-Acevedo et al. 2009
Zacatecas	700.0	Central	Trejo-Acevedo et al. 2009

Table 11. Total DDT levels in serum (ng/g lipid) of children living in different Mexican Regions.

A monitoring program of DDTs in adipose tissue is needed in order to assess the body burden, now that in Mexico this insecticide has been eliminated from the malaria program. However, due to ethical constraints, it is not always possible to obtain adipose tissue samples form healthy individuals. Therefore, alternative matrices are needed; for example, a good correlation between adipose tissue concentration and levels in human milk [50] or human serum [51] has been reported .When the geometric DDE levels in lipid bases are used for the estimation of the adipose tissue/serum DDE ratio, a value near unity is obtained [51].

5. Health effects

DDT and its metabolites have been associated with neurological effects (Dorner and Plagemann 2002; Fenster et al. 2007; Torres-Sánchez et al. 2007; Rocha-Amador et al. 2009), asthma (Sunyer et al. 2006), immunodeficiency (Dewailly et al. 2000; Vine et al. 2000; Vine et al. 2001; Belles-Isles et al. 2002; Bilrha et al. 2003; Cooper et al. 2004; Dallaireet al. 2004), apoptosis (Pérez-Maldonado et al. 2004) and DNA damage in immune cells in children (Yáñez et al. 2004; Herrera-Portugal et al. 2005b).

Location	Total DDT (ng/g lipid)	Region	Reference
Chiapas	12750.0	Southeastern	Yañez et al. 2002
Oaxaca	8050.0	Southeastern	Yañez et al. 2002
Tabasco	8700.0	Southeastern	Torres-Dosal et al. 2011
Mexico, DF	20.0	Central	Lopez-Carrillo et al. 1997
Veracruz	4500.00	Southeastern	Waliszewski et al. 2000
Morelos	20.0	Central	Lopez-Carrillo et al. 2001
San Luis Potosí	1715.0	Central	Yañez et al. 2004

Table 12. Total DDT levels in serum (ng/g lipid) of adults living in different Mexican Regions.

Location	Total DDT	Region	Reference
Coahuila	18400.0	Central	Albert et al. 1980
Mexico DF	6100.0	Central	Albert et al. 1980
Puebla	2700.0	Central	Albert et al. 1980
Veracruz	10000.0	Southeastern	Waliszewski et al. 1996
Veracruz	61000.0	Southeastern	Rivero-Rodriguez et al. 1997
Veracruz	5700.0	Southeastern	Waliszewski et al. 2001
Veracruz	2600.0	Southeastern	Waliszewski et al. 2010
Puebla	800.0	Central	Waliszewski et al. 2010
Veracruz	1900.0	Southeastern	Waliszewski et al. 2011
Veracruz	1400.0	Southeastern	Herrero-Mercado et al. 2010
Veracruz	900.0	Southeastern	Herrero-Mercado et al. 2011

Table 13. Total DDT levels in Adipose Tissue (ng/g lipid) of adults living in different Mexican Regions.

5.1 Cancer

Although it has been suggested that the estrogenic activity of DDE may be a contributing factor for development of breast cancer in women, levels of these compounds are not consistently elevated in breast cancer patients. It was initially reported that levels of *p*,*p*2-DDE were elevated in breast cancer patients (serum or tissue) versus controls [52].More recent studies and analysis of organochlorine levels in breast cancer patients versus controls

show that these contaminants are not elevated in the latter group [53–56]. The study of occupationally exposed workers has not found clear increased risks for other cancers [57].
Two case-control studies of breast cancer have been carried out in Mexico City, with conflicting results. The first study, conducted by Lopez-Carrillo et al. [58] in Mexico City, compared 141 cases of breast cancer with 141 age-matched controls. All subjects were identified at three referral hospitals between March 1994 and April 1996. The arithmetic mean of serum DDE in lipid basis was 562 ppb±676 for the cases and 505 ppb±567 for the controls.The age-adjusted odds ratios for breast cancer regarding the serum level of DDE were 0.69 (95% confidence interval, 0.38–1.24) and 0.97 (CI, 0.55–1.70) for the contrasts between tertile 1 (lowest level) and tertiles 2 and 3, respectively. These estimates were unaffected by adjustment for body mass, accumulated time of breast-feeding and menopause, and other breast cancer risk factors. These results do not lend support to the hypothesis that DDT is causally related to breast cancer. The second study conducted by Romieu et al. [59] compared 120 cases and 126 controls, selected from six hospitals in Mexico City, from 1989 to 1995. Serum DDE levels in lipid basis were higher among cases (mean=3840 ppb±5980) than among controls (mean=2510 ppb±1970).After adjusting for age, age at menarche,duration of lactation, Quetelet index, and serum DDT levels, serum DDE levels were positively r elated to t he risk for b reast cancer (adjusted O R_{Q1-Q2}=1.24), 382 F. Díaz-Barriga et al. (CI, 0.50–3.06; OR_{Q1-Q3}=2.31, 95 percent, CI, 0.92–5.86; OR_{Q1-Q4}=3.81, CI, 1.14–12.80). The increased risk associated with higher serum DDE levels was more apparent among postmenopausal women (OR_{Q1-Q4}=5.26, 95%, CI, 0.80–34.30). Serum DDT level was not related to the risk for breast cancer. In addition to the differences in the comparison of cases and controls, the difference in the serum DDE levels among the women studied is remarkable. Participants from both studies came from similar hospitals, and there were no apparent differences between case and control selection that could explain this divergence. Differences in laboratory procedures is the most feasible explanation.

5.2 Endocrine disruption

DDT is known to have adverse effects on wildlife via endocrine disruption.Clear effects include thinning of the eggshell, feminization, reproduction impairment and development effects [60]. In Mexico two studies in humans have reported findings in this area. Gladen and Rogan [61] found that DDE might affect women's ability to lactate in a study conducted in an agricultural town in northern Mexico. Two hundred and twenty-nine women were followed from childbirth until weaning or until the child reached 18 months of age.DDE was measured in breast milk samples taken at birth, and women were followed to see how long they lactated.Median duration was 7.5 months in the lowest DDE group and 3 months in the highest. The effect was confined to those who had lactated previously – but not for first pregnancies – and it persisted after statistical adjustment for other factors. Rodriguez et al. [62] conducted a study aimed at determining the capability of long-term exposure to DDT of altering the normal endocrine function of the hypothalamus-hypophysis-gonads axis in humans. This included 70 workers dedicated to control malaria in the State of Guerrero,Mexico. The main activities of these workers were the application of pesticides,detection of malaria cases and promotion of preventive measures for control vectors. The average time of exposure to technical grade DDT was 25 years (range: 4–35), their last exposure being 5 months before sampling. An interview gathered information on the occupational history, reproductive performance, life styles and other relevant factors. Blood and urine samples were collected to measure serum levels of DDT and metabolites as

well as levels of LH, FSH, prolactine, and testosterone. Participants ranged in age from 22 to 69 years, and had been employed in the sanitation campaign from 4 to 37 years. Ninety-seven percent of the participants were sprayers of DDT at some time in their occupational history, and 15% are current sprayers. Average levels of DDT and metabolites expressed as μg/g of extractable lipids were: total DDT, 60.1; *p*,*p*2-DDE, 37.41; *p*,*p*2-DDT, 21.52; *p*,*p*2-DDD, 1.07 and *o*2*p*-DDT 0.11. Results show a positive association of LH and FSH with DDT metabolites. An increase of 10 *m*g/g of *p*,*p*2-DDE was associated with an increase of 1.95 UI/L in LH (p=0.01); and 1.10 UI/L per each 10 *m*g/g of *p*,*p*2-DDT (p=0.02). FSH increased 1.09 UI/L per each 10 *m*g/g of *p*,*p*2- DDT (p=0.03). There was a negative association of DDE with testosterone, DDT in Mexico 383 especially for those participants under 55 years of age. These associations suggest direct toxicity to the testicles, especially the Leydig cells, as observed with antineoplasic drugs.

5.3 Genotoxicity

Some studies have reported genotoxic effects in humans heavily exposed to DDT [8]. Therefore, this area has been studied in Mexico. Studies were done in workers from the control program of malaria, and in women living in malarious areas. Herrera et al. [63] evaluated chromosomal translocations in a sample of the above-mentioned workers.Nineteen male sprayers (median age 46 years; range: 22–64),working in campaigns to control malaria vectors in the state of Guerrero, Mexico were included in this study.DDT data obtained in a previous study from eleven individuals, 5 women and 6 men, living in Mexico City, and occupationally unexposed to DDT were used as reference group. Chromosomal aberrations in lymphocytes were analyzed with a chromosome painting technique, a highsensitivity technique for detecting complex chromosomal translocations. *o*,*p*2- DDT was the only isomer significantly associated with the frequency of chromosomal translocations (p=0.003). Individuals presenting the higher levels of *o*,*p*2-DDT in serum (>0.79 *m*g/g fat; n=4) had a mean frequency of chromosomal translocations (5.1¥1000 metaphases), two times higher than that observed in workers occupationally exposed to 0.5 Gy of radiation.A positive relationship between the duration of exposure to DDT, measured as years working for the vector control program, and chromosomal translocations was observed (Fig.2). These results suggest an increased risk for diseases with a genetic component, such as cancer.

Yañez et al. [64] evaluated the association of blood DDT levels and DNA damage using the single cell gel electrophoresis assay. A group of 53 postpartum women were selected from two different areas in San Luis Potosí to assure different exposure levels, one with antecedents of malaria and DDT spraying and the other without malaria. Mean and range levels of DDT, DDE and DDD in whole blood were 5.57 ppm (0.02–20.69), 6.24 ppm (0.04–39.16) and 1.16 ppm (0.01–5.63), respectively. The significant correlation of DNA damage, measured as DNA migration with the logarithm of DDT,DDE and DDD was 0.60, 0.62 and 0.43, respectively. Fig. 3 shows the shape of the association of DNA migration with DDE concentration in whole blood, as obtained by the regression: DNA migration= 71.58+7.62(logDDE), this association was not modified by age, smoking habits, nutrition or occupation. This observational finding in the epidemiological study with postpartum women was reevaluated in an in vitro study.Human blood cells were exposed to three doses of DDT, DDD and DDE.DNA damage was assessed by two different techniques: single-cell electrophoresis and flow cytometry. Results obtained by either technique showed that DNA damage was induced by the three organochlorides and a dose-response was observed with

DDT. The data suggest a DDT-induced DNA fragmentation, and this outcome was also observed with DDE and DDD.

6. References

[1] Stapleton DH (1998) Parassitologia 40 :149
[2] North American Commission for Environmental Cooperation (1998) North American Regional Action Plan on DDT. In: The Sound Management of Chemicals Initiative. Regional Commitments and Action Plans.Montreal, Canada, p 67
[3] Fernández de Castro J (1988) Panorama histórico y epidemiológico del paludismo en México. Secretaría de Salud,México
[4] Implementation Plan to Control Malaria with Alternatives Methods to DDT in Mexico 1999–2001.Government of Mexico, January 1999
[5] ISAT, 2002. Diagnóstico situacional del uso de DDT y el control de la malaria. Informe regional para México y Centroamérica. Instituto Salud Ambiente y Trabajo, México. <http://www.cec.org/files/PDF/POLLUTANTS/InfRegDDTb_ES_EN.pdf> (accessed 07.07.09.).
[6]ATSDR (2010) Toxicological Profile for DDT,DDE, and DDD.Agency for Toxic Substances and Disease Registry,Atlanta,Georgia
[7] Zeng EY,Yu CC, Tran K (1999) Environ Sci Technol 33:39
[8] Fisher SW (1995) Rev Environ Contam Toxicol 142:87
[9] Albert LA (1996) Rev Environ Contam Toxicol 147:1
[10] Lu FC (1995) Reg Toxicol Pharmacol 21 :352
[11] Woodruff T,Wolff MS, Davis DL,Hayward D (1994) Environ Res 65:132

6

Presence of Dichlorodiphenyltrichloroethane (DDT) in Croatia and Evaluation of Its Genotoxicity

Goran Gajski[1], Marko Gerić[1], Sanda Ravlić[2], Željka Capuder[3] and Vera Garaj-Vrhovac[1]
[1]*Institute for Medical Research and Occupational Health*
[2]*Ruđer Bošković Institute, Center for Marine Research*
[3]*Dr. Andrija Štampar Institute of Public Health*
Croatia

1. Introduction

Pesticide is any substance or mixture of substances used for preventing, destroying or controlling any pest. According to the Food and Agriculture Organization (FAO) pest is defined as an organism that: (1) is a vector of human and animal disease; (2) can interfere with or cause harm to any step in food, wood and agricultural goods production, transport and storage; (3) can lower the quality of animal food or drugs. Extended definition of pesticide includes all substances: (1) used as plant growth regulator, defoliant and desiccant or agent for prevention of premature fruit fall; (2) applied to crops in order to protect them from deterioration during storage and transport (Food and Agriculture Organization [FAO], 2005). Every pesticide consists of active ingredient whose main function is to cause harm on the pest and inert carrier substance to improve storage, handling, application, efficiency and safety of the pesticide (World Health Organization [WHO], 1990).

For easier dealing with large numbers of pesticides, there are several classifications which divide pesticides in some classes. According to the chemical structure pesticides can be classified as: pyrethroids, carbamates, thiocarbamates, dithiocarbamates, organophosphates, organochlorides, phenoxy and benzoic acid herbicides, triazines, triazoles, ureas etc. (Kamir, 2000). Another classification is done considering the target organism, so pesticides can be classified as: insecticides, fungicides, herbicides, virucides, avicides, molluscicides, nematocides, rodenticides etc. (WHO, 1990). It is important to notice that pesticide's mode of action is not restricted just to the target organism; it also has negative effects on other organisms. That is why the World Health Organization (WHO) recommends the classification of pesticides by hazard. The base of this classification is determining the lethal dose (LD_{50}) for 50% of rat population after acute oral and dermal pesticide exposure. Depending of the LD_{50} value, as presented in Table 1, pesticides are classified as extremely, highly, moderately or slightly hazardous (WHO, 2009a).

It can be assumed that fighting against the pests started when human civilization was introduced to agriculture which ensured enough food for further development. The first

known historical use of pesticide was observed in Mesopotamia 2500 BC. People in ancient Sumer used sulphur to treat crops thus killing pests (Miller, 2004). Around 500 BC ancient Greek historian Herodotus described the use of castor-oil plant as a mosquito repellent. People in Egypt who lived in swampy areas lit the castor-oil laps which had an unpleasant smell. Also they fixed fishing nets around their beds before going to sleep. This information suggests that ancient Egyptians used similar techniques to deal with mosquitos as today's insecticide-treated nets (Charlwood, 2003; WHO, 2007). After long period of natural pesticides use (e.g. arsenic, mercury, nicotine sulphate, pyrethrum, etc.), in 20th century the revolution of synthesized pesticides started.

Class	LD_{50} for rats (mg/kg of body mass)				
		Oral		Dermal	
		Solids	Liquids	Solids	Liquids
I a	Extremely hazardous	5 or less	20 or less	10 or less	40 or less
I b	Highly hazardous	5 – 50	20 – 200	10 – 100	40 – 400
II	Moderately hazardous	50 – 500	200 – 2000	100 – 1000	400 – 4000
III	Slightly hazardous	over 500	over 2000	over 1000	over 4000

Table 1. The WHO pesticide classification based on LD_{50} (adapted from WHO, 2005).

DDT (1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane) was one of the most used pesticides in the mid 20th century. This organochloride was synthesized in 1874 by Othmar Zeidler and its pesticides properties were discovered in 1939 by Paul Hermann Müller who was awarded Nobel Prize in Physiology or Medicine for this discovery (Agency for Toxic Substances and Disease Service [ATSDR], 2002; Turusov et al., 2002). Commercially used DDT is a mixture of several isomeric forms: *p,p'*-DDT (85%), *o,p'*-DDT (15%), *o,o'*-DDT (trace amounts) and its breakdown products DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene) and DDD (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane). The basic chemical properties of *p,p'*-DDT, *p,p'*-DDE and *p,p'*-DDD are shown in Table 2. According to WHO, DDT is moderately hazardous pesticide with LD_{50} of 113 mg/kg (WHO, 2009a).

In the period of almost 35 years, 2 million tons of DDT was used to control malaria and typhus, thus contaminating water, soil and air. When DDT and its metabolites enter the environment they have the potential to stay adsorbed in the sediment for more than 100 years. The best example of DDT's persistency is that it has been found in the areas 1000 km off the spraying spot and even in the Arctic animals and ice where it has never been used (ATSDR, 2002). One of the most popular acts that contributed global DDT ban in 1973, was Rachel Carson's book *Silent Spring* written in 1962 where she emphasized the problem of the DDT use (Carson, 2002). Today DDT is still legally produced (China, India, and North Korea) and used in the countries of the third world (e.g. India, Ethiopia, South Africa etc.) for malaria control (WHO, 2009b).

There are four possible ways for DDT to enter organisms: (1) ingestion, (2) inhalation, (3) dermal exposure, and (4) placental transport. Once in the body DDT is metabolized mainly in the liver and partly in the kidneys to its most familiar metabolites DDE and DDD. Detailed metabolic pathways are shown in Figure 1. Conjugated forms of 2,2-bis(4-chlorophernyl)-acetic acid (DDA) are then excreted through urine and faeces. The most dangerous property of DDT is that it possesses high bioaccumulation and biomagnification potential. As the trophic level rises, the concentration of DDT and/or its metabolites increases. All three compounds are reported to be harmful to either human or animals

(Brooks, 1986; Gold & Brunk, 1982, 1983; Morgan & Roan, 1974; Peterson & Robinson, 1964). As mentioned in the book *Silent spring*, DDT (especially metabolite DDE) has negative effects on bird's reproductive system by reducing Ca2+ transport which results with eggshell thinning thus increasing lethality (Carson, 2002).

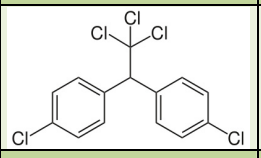
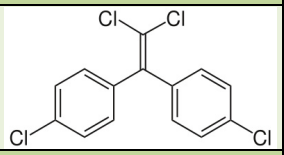
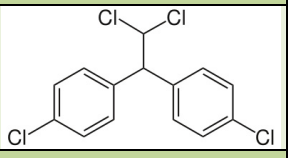
	p,p'-DDT	*p,p'*-DDE	*p,p'*-DDD
Chemical formula	$C_{14}H_9Cl_5$	$C_{14}H_8Cl_4$	$C_{14}H_{10}Cl_4$
Chemical structure			
Molecular mass	354.49	318.03	320.05
Physical state	Solid[a]	Cristalline solid[a]	Solid[a]
Colour	Colourless crystals, white powder	White	Colourless crystals, white powder
Melting point	109 °C	89 °C	109 – 110 °C
Boiling point	Decomposes	336 °C	350 °C
Solubility in water	0.025 mg/L[b]	0.12 mg/L[b]	0.09 mg/L[b]
Solubility in organic solvents	Slightly soluble in ethanol, very soluble in ethyl ether and acetone	Lipids and most organic solvents	NA*

Table 2. Chemicals properties of *p,p'*-DDT, *p,p'*-DDE, and *p,p'*-DDD (adapted from ATSDR, 2002), [a] room temperature, [b] at 25°C, *NA – not available.

Not only birds are affected by this persistent pollutant, animals with higher amounts of fatty tissue have high levels of DDT and its metabolites (e.g. polar bears, orcas, belugas etc. (Crinnion, 2009; Galassi et al., 2008; Glynn et al., 2011; Okonkwo et al., 2008)). Although the acute DDT poisoning is rare, there are reports of negative effects on human health when exposed to low concentrations of DDT and its metabolites for longer periods. As shown in animals, the major damage was done on reproductive system where DDT can interfere with reproductive hormones. Also, there have been some reports regarding neurotoxicity, hepatotoxicity, imunotoxicity and genotoxicity (ATSDR, 2002). According to International Agency for Research on Cancer (IARC) DDT is classified as possible carcinogen to humans (IARC, 2009) and the increase in frequency of some cancers was detected (e.g. breast, testicular cancer etc.; Aubé et al., 2008; McGlynn et al., 2008).

As mentioned above, DDT introduced revolution in control of vector-borne diseases. Today, there are reports that some *Anopheles* species are resistant to DDT (Morgan et al., 2010) which implies that new methods of fighting vectors should be introduced. According to WHO one can observe the decrease in both number of countries using pesticides to control

vectors (from 72 countries in 2006 to 61 countries in 2007) and in total amount of used DDT for vector control (from 5.1 million kg in 2006 to 3.7 million kg in 2007). Also WHO stimulates the import of new technologies to improve efficiency of insecticide-treated nets and long-lasting insecticidal nets (WHO, 2007). The importance of introducing new technologies for non harmful and efficient global pest fighting can be seen in estimation that only in United States, environmental and social costs of pesticide use are 9645 million dollars per year (Pimentel, 2005).

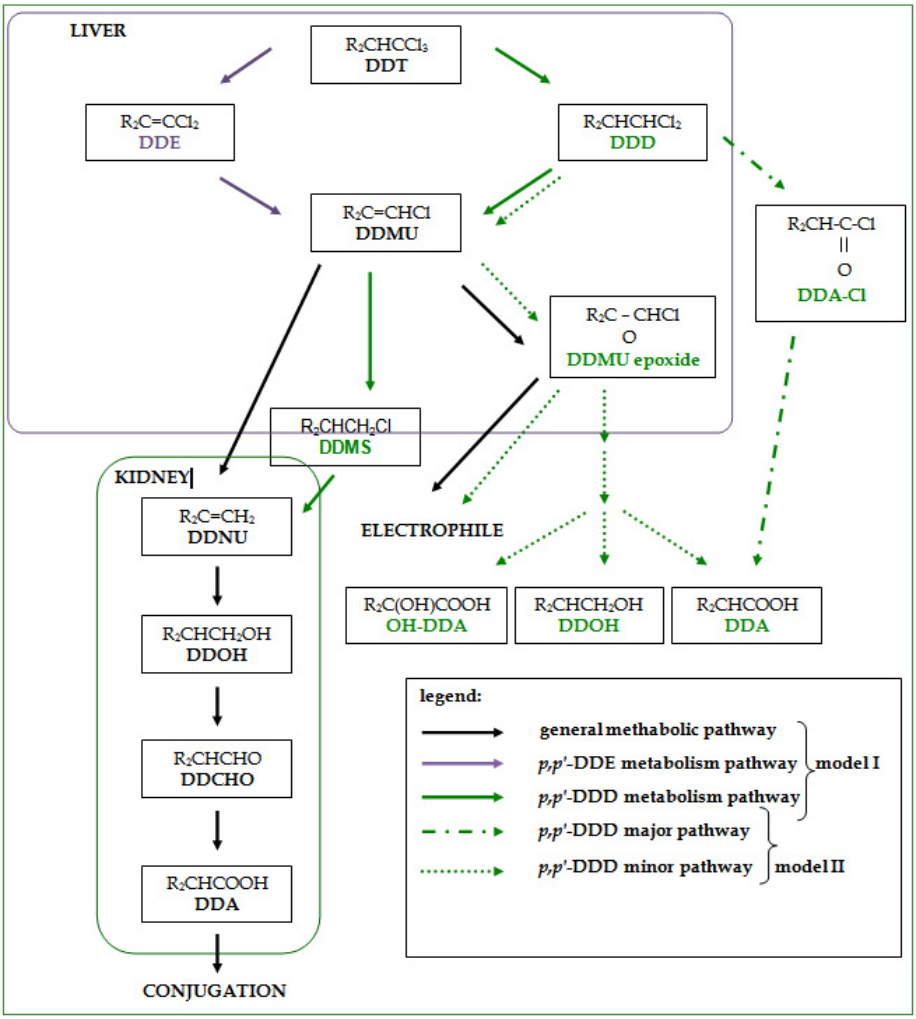


Fig. 1. Models of DDT metabolic pathways (adapted from Gold & Brunk, 1982 and Peterson & Robinson, 1964).

2. Presence of DDT in Croatia

2.1 Production and use in Croatia

Croatia, as a Mediterranean country, has been affected by malaria since the ancient times, especially in the coastal area. Only areas along the Velebit and Biokovo and the districts Hvar and Supetar have been spared from the disease. Most exposed to malaria have been the cities Nin, Benkovac, Obrovac, Skradin, Knin, Drniš, Šibenik, Imotski, Vrgorac and Metković area, the valley of the Neretva River, where malaria was called *Morbus naronianus* - Neretva disease. In 1820 French pharmacists Pierre Joseph Pelletier & Jean Biename Caventou isolated the alkaloid quinine, while doctor Lujo Adam from the island Lošinj was probably the first in the world who injected quinine sulphate into the veins of people sick from malaria. In 1902 doctor Rudolf Battara conducted the first controlled study of drug quinine prophylaxis on the overall population in Nin. The success of the study encouraged implementation of this prophylaxis in other places in Dalmatia in the upcoming years (Dugački, 2005).

DDT was first used in Croatia in 1941, when spraying was carried out against the lice of people as part of educational campaigns in the School of Public Health. The first mass use of DDT was carried out on the front of Srijem in 1945 by spraying soldiers as prevention of typhoid fever with the preparation "neocid", which was donated by the International Red Cross (Bakić, 2011). Companies Chromos from Zagreb and Zorka from Šabac, with their preparations Pantakan and Pepein, were the first producers of DDT in Croatia and the former Yugoslavia. Since 1946 DDT was produced in the form of dust, and since 1949 in the form of concentrated emulsions (Table 3). Production of pesticides in Croatia was small, but large quantities of pesticides were imported. Data on quantities of imported pesticides does not exist, except for the year 1957, when active substances for plant protection products in quantities of 1435 tons were imported. The reason for the continuing production decline of DDT was the lack of foreign exchange quotas for the purchase of organic substances necessary for the production of pesticides. Production and use of DDT in Croatia was until 1972, when its use was banned in agriculture, while in forestry DDT was still used until 1984 (Table 4; National Implementation Plan [NIP], 2004).

Year and purpose	Type of pesticide	Amount (t)	Note
1958. production	DDT	2150	1600 t sprayer concentrate
1959. plan	DDT	3000	550 t emulsifiable concentrate
1959. consumption	DDT	3122	
	DDT + Lindane	687	
1963. production	DDT	2327	
	DDT + Lindane	1854	4.6-6.6 % DDT + 0.3-0.7 lindane content of active substance in preparation

Table 3. The planned and produced quantities of DDT, according to the available data for Yugoslavia in some years (adapted from Hamel, 2003).

Year	Consumption of DDT in forestry (kg/year)	Consumption of DDT in agriculture (kg/year)
1963.	2312	-
1964.	-	1784
1965.	53428	1196
1966.	280	14051
1967.	-	16323
1968.	600	4183
1969.	-	6051
1970.	-	5450
1971.	2363	4296
1972.	4912	1078
1973.	884	0
1974.	8437	0
1975.	6907	0
1976.	8437	0
1979. – 1987.	18658	0

Table 4. Consumption of DDT in kg per year in agriculture and forestry in Republic of Croatia (adapted from NIP, 2004).

Today, list of active substances permitted for use in the Republic of Croatia is synchronized with the official list of active substances permitted in the means of the European Union. The regulations require from pesticide manufacturers to provide data on all possible risks to human health and the environment, as well as data on the effectiveness of pesticides and information on possible contamination in order to obtain licenses for the production and use.

2.2 Methodology

Residues of DDT and its metabolites were analyzed in samples of surface water, soil and food. One of the most sensitive techniques for measuring the rest of DDT and its metabolites in samples from the environment is gas chromatography with electron capture detector (GC/ECD). DDT and its metabolites were determined by applying analytical methods or modifications of the method: International Organization for Standardisation [ISO] 6468 (2002), ISO 10382 (2002), Reference Methods for Marine Pollution (United Nations Environment Programme/International Atomic Energy Agency [UNEP/IAEA], 1982) and EN 1528 1-4 (1996), according to the scheme; extraction, purification of the extracts and quantitative analysis.

2.2.1 Sample extraction and cleanup

Water samples, two to four litres, were extracted with methylene chloride (HPLC grade for spectroscopy, Merck, Darmstadt, Germany) as solvent using an Ultra Turrax system. Extracts were dried over granular anhydrous sodium sulphate and concentrated using rotary evaporator (ISO 6468, 2002). The soil sample was sieved (<2 mm) and stored at room temperature for two days before the experiments. 10 g soil sample (dry matter) was extracted in an ultrasonic bath (ISO 10382, 2002) and the extract was dried by passing them through anhydrous sodium sulphate. Determination of DDT in food samples was based on

method EN 1528 1-4 (1996). Approximately 10-20 g of food were homogenized and extracted with hexane, the extract was evaporated in a rotary evaporator and in nitrogen stream to ensure dryness. Contents of fat were determined weighing a dry sample. Milk fat portions were extracted from each individual sample of milk, cheese, cream and yoghurt according to the method by Sannino et al. (1996). Butter does not normally require extraction procedures. An automated gel permeation chromatographic (GPC) procedure was used to determine DDT residues in fatty foods. About three grams of the fat or less was dissolved into methylene chloride and cleaned up by GPC with a Biobeads SX3 column (OI Analytical, College Station, TX, USA) and a methylene chloride as eluant. About 10 g of fish tissue was weighed, homogenized with anhydrous sodium sulphate and extracted with pesticide grade hexane (Merck, UNEP/IAEA, 1982). The extract was condensed in a rotary flask vaporator to a specific aliquot (5 ml). The aliquot was then subjected to acid treatment by adding concentrated sulphuric acid (Merck). All samples were cleaned up with florisil, commercially available cartridges, 3-6 mL, 500-1000 mg (Kodba & Voncina, 2007). Extracts were concentrated using evaporator in nitrogen stream. An aliquot of each extract was transferred to vials for the quantitative analysis.

2.2.2 Gas chromatograph analysis

The samples were analyzed by gas chromatograph (GC) Shimadzu (Models GC 2010 and GC 17A series, Tokyo, Japan) equipped with autosampler and electron capture detector (ECD) on two fused silica capillary columns of different polarity. Nitrogen was used as the carrier gas with a flow rate of about 1.4 mL/min and as the makeup gas. The injection was set at splitless mode. The injection port and detector temperature were 250 and 300 ^{0}C, respectively. The compounds *p,p*'-DDE, *p,p*'-DDD, *o,p*'-DDT and *p,p*'-DDT were identified by comparing peak retention times between samples and known standards. The standard samples of the 18 pesticides were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany, and AccuStandard Inc., New Haven, CT, USA) with the purities of 97-99%. Calibration standard curves were created and DDT residues were quantitatively determined by comparison of the retention times and peak areas of the sample chromatogram with those of standard solutions run under the same operating conditions. Peak confirmation was done by running the samples and the standard on another column and comparing. The concentrations of DDT residues in each sample were reported as ng/L or µg/kg. Limit of quantification for every matrix is presented in Table 5. The laboratory has participated (annually) in intercalibration study proficiency test.

Limit of quantification					
		p,p'-DDE	*p,p'*-DDD	*o,p'*-DDT	*p,p'*-DDT
Water	µg/L	0.0005	0.001	0.001	0.001
Soil (dry mater)	µg/g	0.002	0.003	0.004	0.003
Milk (liquid) and milk products (milk fat)	µg/g	0.0001	0.0004	0.0004	0.0004
Fish and fish products (wet weight)	µg/g	0.0005	0.001	0.0006	0.001
Food (fat)	µg/g	0.001	0.002	0.004	0.002

Table 5. Limit of quantification for *p,p'*-DDE, *p,p'*-DDD, *o,p'*-DDT and *p,p'*-DDT.

2.3 Results

During his lifetime a human being has been exposed to DDT and other pesticides through food, water and the environment. A level of these exposures can be established by analysing of samples from the environment. All food samples come from the market in Croatia while samples of surface water and soil were from different areas of Croatia. Food, particularly dairy products, meat and fish, has been identified as the primary immediate intake route of DDT and other organochlorine pesticides for the population (Johansen et al., 2004).

Samples of surface water of the rivers Sava, Krka, Mrežnica, Kupa, Zrmanja and Cetina were analyzed. The concentration of total DDT in surface waters in Croatia for the period 2009-2010 amounted from below detectable limit to 0.021 μg/L, with median value 0.0016 μg/L (Table 6). The average concentrations of total DDT in surface waters were 0.0026 μg/L. These results comply with those reported by Drevenkar et al. (1994), Drevenkar & Fingler (2000), and Fingler et al. (1992) in their papers. DDT may reach surface waters primarily by runoff, atmospheric transport and drift, or by direct application (e.g. to control mosquito-borne malaria). DDT is practically insoluble in water; but some DDT may be adsorbed onto the small amount of particulate matter present in water (ATSDR, 2002).

The concentration of DDT and metabolites in analyzed samples of soil were below 0.5 μg/g of dry matter (legally prescribed limit values). These analyses were conducted on the samples collected over the last two years and only from a few locations and therefore cannot be found sufficient to make any kind of general conclusions about the current situation. There is no systematic monitoring of DDT in soil in Croatia, resulting with little relevant data. Studies such as Picer et al. (2004) are rare examples of organized research in this area in Croatia, conducted as a part of post war damages in areas were there were concerns that soil was contaminated.

Data on levels and distribution of DDT and other persistent organic pollutant (POPs) in surface waters and soils in Croatia is insufficient, despite these hydrophobic substances being extremely important for assessing environmental contamination. DDT compound in sample of fish tissue were present in very low concentrations, although it is well known that DDT bioaccumulates in marine species. The mean value of DDT in fish tissue, analyzed in year 2007, was 3.8 μg/kg wet weights, with median value of 4.7 μg/kg . Similar results are reported by Krautchaker & Reiner (2001), and Bošnir et al. (2007). The five dairy products have been examined for the rest of DDT: milk, butter, cheese, cream and yoghurt. The mean values of the residual concentrations of total DDT in the examined dairy products were 29.6 μg/kg fat, respectively, with median 25.2 μg/kg (Table 6). The presence of DDT in milk and milk products has also been reported by Krautchaker & Reiner (2001) and Bošnir et al. (2010) as well as in other countries (Nevein et al., 2009; Dawood et al., 2004; Bulut et al., 2010). These studies have found that DDT complex were the most frequent contaminants in dairy products. Heck et al. (2006) concluded there is no difference in DDT in raw and pasteurized milk. The concentration of DDT in meat products sampled at food markets was 1.2-740.0 μg/kg fat, with median value 16.2 μg/kg fat (Table 6). These results should be taken with caution since the origin of meat is unknown; whether the meat is from domestic production or imported from other countries of the world. Meat is imported primarily from developing countries where there is limited or no control over the use and/or control of pesticide residues in foods. These results comply with those reported by Krautchaker & Reiner (2001), Covaci et al. (2004), and Tompić et al. (2011).

	Value range	Mean	Median
Surface and ground water (ng/L)	0.6 – 20.5	2.6	1.6
Soil (µg/g; dry matter)	0 – 0.005	0.002	0.002
Fish and fish products (µg/kg; wet weight)	0.2 – 31.0	3.8	4.7
Meat and meat products (µg/kg; fat)	1.2 – 74.0	51.7	16.2
Milk and milk products (µg/kg; milk fat)	11.3 – 79.9	29.6	25.2

Table 6. Concentrations of DDT compounds in different samples.

Lamb meat results (Table 7) demand special attention, since the concentrations of *p,p'*-DDE were generally high. Similar results were found by Tompić et al. (2011) in samples of lamb imported from Bulgaria. *p,p'*-DDE are found in every examined sample of lamb meat, which indicates the need for continuous monitoring of concentrations of this metabolites in samples of lamb meat.

	p,p′-DDE (µg/kg)	p,p′-DDD (ng/g)	p,p′-DDT (ng/g)	Total DDT (ng/g)	Total DDT (µg/kg)
Lamb meat	739.0	-	0.4	74.3	743.0
Lamb meat	173.0	-	0.5	17.8	178.0
Lamb meat	609.0	0.6	-	1.5	615.0
Lamb meat	964.0	6.1	1.2	103.7	1037.0
Lamb meat	354.0	1.4	0.5	37.3	373.0

Table 7. Concentrations of DDT compounds in samples of lamb meat.

Generally, it was observed that the total DDT or its metabolites residues were bellow acceptable and legally prescribed boundaries. These results highlight the need for regular analyzing of a larger number of samples from the environment to DDT residues and other chemicals of POPs, especially in imported food.

3. Cytogenetic methods for detection of pesticide genotoxicity

Pesticide exposure is ubiquitous, due not only to agricultural pesticide use and contamination of foods, but also to the extensive use of these products in and around residences. Because of their biological activity, the use of pesticides may cause undesired effects to human health. Pesticides tend to be very reactive compounds that can form covalent bonds with various nucleophilic centers of cellular biomolecules, including DNA (Crosby, 1982). For instance, the induction of DNA damage can potentially lead to adverse reproductive outcomes, the induction of cancer and many other chronic diseases (Ribas et al., 1996; Lander et al., 2000; Meinert et al., 2000; Ji et al., 2001). A great variety of tests and test systems based on microbes, plant and animals have been developed in order to asses the genotoxic effects of xenobiotic agents, including pesticides. Biomonitoring studies on human populations exposed to pesticides are employing circulating lymphocytes as biomarkers of exposure (and perhaps of effect). Those studies have essentially focused on cytogenetic end-points such as chromosomal aberrations (CA), sister-chromatid exchanges (SCE) and micronuclei (MN) frequency. Genetic damage at the chromosomal level entails an alternation in either chromosome number or chromosome structure, and such alternations can be measured as CA or MN frequency. The SCE analysis was also adopted as an indicator of genotoxicity, although the exact mechanism that leads to an increased exchange

of segments between sister chromatids is not known in detail at present (Palani-Kumur & Panneerselvam, 2008). Recent studies revealed the nucleotide pool imbalance can have severe consequences on DNA metabolism and it is critical in SCE formation. The modulation of SCE by DNA precursors raises the possibility that DNA changes are responsible for the induction of SCE and mutations in mammalian cells (Popescu, 1999; Ashman & Davidson, 1981). While increased levels of CA have been associated with increased cancer risk (Hagmar et al, 1994, 1998), a similar conclusion has not been reached for SCE or MN. However, high levels of SCE and MN frequency have been observed in persons at higher cancer risk due to occupational or environmental exposure to a wide variety of carcinogens (Fučić et al, 2000; Vaglenov et al, 1999; Fenech et al, 1997). Evidence of CA increases, mainly as structural chromosomal aberrations in occupationally exposed populations. The sensitivity of SCE is lower than that of the CA test in detecting genotoxic effects related to pesticide exposure and fewer data are therefore, available for MN than for the other cytogenetic endopoints (Bolognesi, 2003). Exposure to potential mutagens or carcinogens can provide an early detection system for the initiation of cell disregulation. Biomarkers of effect are generally pre-clinical indicators of abnormalities and the most frequently used in genotoxicity assessment are comet assay and cytokinesis-block micronucleus test that are being proposed as a useful biomarkers for early effects. The cytogenetic endpoints can give indication of genetic damage; hence they are used as effective biomarkers of exposure *in vivo* and *in vitro*. In recent years, the comet (single-cell gel) assay has been established as a useful technique for studying DNA damage and repair (Tice, 1995). The comet assay combines the single-cell approach typical of cytogenetic assays with the simplicity of biochemical techniques for detecting DNA single strand breaks. The advantages of the comet assay include its simple and rapid performance, its sensitivity for detecting DNA damage, and the use of extremely small cell samples (Hartmann et al., 1998). The advantage of micronucleus assay is its simplicity and speed over the assay of chromosomal aberration. Both techniques have become an important tool for genotoxicity testing because of their simplicity of scoring and wide applicability in different cell types. These techniques became the methods of choice for studies of environmental and occupational exposure to air pollutants, metals, radiation, pesticides, and other xenobiotics.

3.1 Comet assay

The comet assay, also known as the single-cell gel electrophoresis assay (SCGE), is a method for detecting DNA strand breakage (single-strand DNA breaks, alkali-labile sites, double-strand DNA breaks, incomplete repair sites, and inter-strand cross-links) in virtually any nucleated cell (Collins et al., 2004, 2008; Shaposhnikov et al., 2008).

First quantification of DNA damage in individual cells was done by Rydberg & Johanson (1978). After gamma–irradiation they embedded cells in agarose on microscopic slides and lysed under mild alkali conditions. Upon neutralization, the cells were stained with acridin orange and the extent of DNA damage was measured by the ratio of green (indicating double–stranded DNA) to red (indicating single–strand DNA) fluorescence. To enhance the sensitivity for the DNA damage detection, Östling & Johanson (1984) proposed that strand breaks would enable DNA loops to stretch out upon electrophoresis, so the microelectrophoretic procedure under pH of 9.5 was developed. As reported by Singh et al. (1988) this pH of 9.5 is below the limit for DNA unwinding, and was notify to detect only double strand breaks (DSB), with more strongly alkaline conditions (pH 10 or above) needed for unwinding and detection of single strand breaks (SSB). It has been shown that

neutral or mildly alkaline comet assay has the same limit of detection of DNA damage (SSB) as the alkaline comet assay, although the use of neutral pH does effect the comet image obtained (Collins, 2004). The comet tails are less pronounced at netural pH, and this can be an advantage when a less sensitive method is needed, for example when investigating cells that have large amount of background, or induced damage is high (Angelis et al., 1999).

Alkaline version of the comet assay was presented by Singh et al. (1988) in which DNA is allowed to unwind at pH>13. In their paper DNA damage was measured as the migrating distance of DNA from the nucleoid. In 1990, Olive et al. (1990) also under alkaline conditions developed the concept of the tail "moment", a combination of tail length and DNA content, as a measure of DNA damage. Also, in 1990, the name "Comet assay" was introduced and the application of the first image analysis program was described (Olive, 1989; Olive et al., 1990; Sviežená et al., 2004). Image analysis has become essential for objective measurement of low–dose effects, or for distinguishing small differences among sub–populations of cells. Strong alkaline conditions enabled clearer images, and besides SSBs other types of DNA damage could be detected, such as alkaline labile sites (Tice et al., 2000). Olive et al. (1990) revealed that employing milder alkaline (pH 12.3) conditions prevents conversion of alkaline labile sites into breaks. Under the pH>13 alkali labile sites are formed into SSBs, thus revealing otherwise hidden damage. Therefore, by modifying the pH of lysis and/or electrophoresis over the range of 9.5–13.5, one can apply a comet assay of different sensitivity, but of similar limits of detection (Collins et al., 1997; Angelis et al., 1999; Wong et al., 2005).

In its basic form, comet assay gives limited information on the type of DNA damage being measured. Single strand breaks detected by standard alkaline method are not the most interesting of lesions, because they are quickly repaired, and are not regarded as a significant lethal or mutagenic lesion. Many genotoxic agents do not induce strand breaks directly. They may create apurinic/pyrimidinic (AP) sites, which are alkali labile and are probably converted to breaks while DNA is in the electrophoresis solution at high pH. Furthermore, it is not possible to determine whether the high level of breaks in the comet assay is the indicator of high damage or efficient repair, due to temporary presence of breaks in the lesions repair via base excision or nucleotide excision (Collins et al., 1997).

More recently, the assay was modified further to enable the detection of specific kinds of DNA damage by combining the assay with the use of a purified DNA repair enzymes, which recognize the lesions along the DNA and convert them into the breaks expressed as an increase in comet DNA migration. Briefly, the DNA in the gel, following lysis, is digested with a lesion–specific repair endonuclease, which introduces breaks at sites of damage. In principle, any lesion for which a repair endonuclease exists can be detected in this way. To date, endonucleases most commonly used in the modified comet assay are the bacterial enzymes which recognize different types of oxidative damage. The first enzyme to be used was endonuclease III, a glycosylase which recognizes a variety of oxidized pyrimidines in DNA and removes them, leaving an AP site (Doetsch et al., 1987). Formamidopyrimidine DNA glycosylase (FPG) has the ability to convert altered purines, including 8-oxoguanine, into DNA breaks (Collins, 2007; Dušinská & Collins, 1996). When using these enzymes to measure oxidative DNA damage the usual practice is to incubate a slide with buffer alone in parallel with the enzyme slide. Slide with buffer would be a valid control slide, due to small increase in strand breaks on incubation without enzyme (Collins, 2009; Gajski et al., 2008). Recently, a mammalian analogue of FPG, 8–oxoguanidine DNA glycosylase, or OGG1, has been applied in the comet assay (Smith et al., 2006). OGG1 is the major base extension repair

enzyme that initiates the repair of oxidative base lesion. It is a bifunctional DNA glycosylase possessing both DNA glycosylase and AP lyase activities (Boiteux & Radicella, 2000). In human it is named hOGG1. hOGG1 recognizes both 8-oxoguanine (8-oxodG) and 8-oxoadenine (8-oxodA) and removes these oxidized bases from double-stranded DNA, initiating the base lesion repair process (Smith et al., 2006). 8-hidroxy-2-deoxyguanosine (8-OHdG) lesion causes G→T and A→C transversions (Moriya, 1993) that have been reported as the sites of spontaneous oncogene expression and ultimately cancer manifestation (Valko et al., 2004; Bartsch, 1996; Shinmura & Yokota, 2001). Deletion of the hOGG1 gene was shown to be associated with accumulation of 8-OHdG lesion and increase in mutational risk (Hansen & Kelley, 2000; El-Zein et al., 2010).

For evaluation of DNA specific damage the comet assay has also been coupled with the method of fluorescent in situ hybridization (Comet–FISH). Since its initial development, Comet–FISH has been used to handle a number of quite different questiones. First, it was used to identify chromosome-specific areas on electrostretched DNA fibres and to determine their special distribution (Santos et al., 1997). Further applications were then to detect region–specific repair activities (Horvathova et al., 2004; McKenna et al., 2003; Mellon et al., 1986), genotoxic effects in total DNA and in telomeres (Arutyunyan et al., 2005), or in tumor relevant genes, like TP53 (Schaeferhenrich et al., 2003). Also specific chromosomal alternations (Harreus et al., 2004) were studied as were genetic instabilities (Tirukalikundram et al., 2005). Comet–FISH has also been used to discriminate between DNA double-and single-strand breaks (Fernandez et al., 2001). Whereas results from the Comet assay alone reflect only the level of overall DNA damage, the combination with the FISH-technique allows the assignment of the probed sequences to the damaged or undamaged part of the comet (tail or head, respectively). If two fluorescence signals are obtained with a probe for a particular gene in the head of a comet, this indicates that the gene is in an undamaged region of DNA, whereas the appearance of a spot or several spots in the tail of a comet indicates that a break or breaks has/have occurred in the proximity of the probed gene.

3.2 Micronucleus assay

Human exposure to environmental mutagens can be monitored using cytokinesis–block micronucleus (CBMN) assay (Natarajan et al., 1996) which is an efficient biomarker for diagnosing genetic damage and/or genome instability at the chromosome/molecular level in animal and/or human cells. It provides a comprehensive measure of chromosome breakage, chromosome loss, chromosome rearrangments, non-disjuction, gene amplification, necrosis and apoptosis (Fenech, 2000, 2006; Kirsch-Volders et al., 2000).

In the classical cytogenetic techniques, chromosomes are studied directly by observing and counting aberrations in methaphases (Natarajan & Obe, 1982). The complexity and laboriousness of enumerating aberrations in methaphase and the confounding effect of artefactual loss of chromosomes from methaphase preparations has stimulated the development of a simpler system of measuring chromosome damage. More than a century ago micronuclei were described in the cytoplasm of erythrocytes and were called "fragment of nuclear material" by Howell or "corpuscules intraglobulaires" in the terminology of Jolly in the late 1800s and early 1900s. To the hematologists these structures are known as "Howell-Jolly bodies". Similar structures were described in mouse and rat embryos and in *Vicia faba* (Thoday, 1951) and called "fragment nuclei" or "micronuclei". In the early 1970s the term micronucleus test was suggested for the first time by Boller & Schmidt (1970) and

Heddle (1973) who showed that this assay provided a simple method to detect the genotoxic potential of mutagens after *in vivo* exposure of animals using dividing cell population such as bone marrow erythrocytes. A few years later it was shown by Countryman & Heddle (1976) that peripheral blood lymphocytes could also be used for the *in vivo* micronucleus approach and they recommended using micronuclei as a biomarker in testing schemes. As only dividing cells could express micronuclei, for the *in vitro* micronucleus studies it was necessary to establish cell proliferation and micronucleus induction at the same time. The decisive breakthrough of micronuclei as assay for *in vitro* genotoxicity testing came with work of Fenech & Morley (1986) that developed the CBMN assay. In the CBMN assay, cells that have completed nuclear division are blocked from performing cytokinesis using cytochalasin–B and are consequently readily identified by their binucleated appearance (Fenech & Morley, 1985, 1986). Whereas micronuclei originate from chromosome fragments or whole chromosomes that leg behind at anaphase during nuclear division, their occurrence is proven in binucleated cells. As a consequence, the CBMN assay has been shown to be more accurate and more sensitive than the conventional methods that do not distinguish between dividing and nondividing cells (Fenech & Morley, 1986; Fenech, 1991; Kirsch-Volders & Fenech, 2001).

Baseline or spontaneous micronucleus frequencies in culture human lymphocytes provide an indicator of accumulated genetic damage occurred during the lifespan of circulating lymphocytes. The half-life and mean lifespan of T-lymphocytes has been estimated to be three to four years, respectively (Natarajan & Obe, 1982; Buckton et al., 1967). The observed genetic instability may also reflect accumulated mutations in the stem cell lineage from which the mature lymphocytes originate. The type of mutations that could contribute to spontaneous micronuclei include: 1) mutations to kinetochore proteins, centromeres, and spindle apparatus that could lead to unequal chromosome distribution or whole chromosome loss at anaphase, and 2) unrepaired DNA strand breaks induced endogenously or as a result of environmental mutagens, which may result in acentric chromosome fragments. Studies using kinetochore antibodies to identify whole chromosomes suggest that approximately 50% of spontaneously occurring micronuclei are the consequence of whole chromosome loss and the rest are presumably derived from acentric chromosome fragments (Thompson & Perry, 1988; Fenech & Morley, 1989; Eastmond & Tucker, 1989). The spontaneous micronucleus frequency refers to the incidence of micronucleus observed in the absence of the environmental risk or exposure that is being assessed. The spontaneous micronucleus frequency of a population has to be established to determine acceptable normal values as well as providing baseline data for those situations when spontaneous micronucleus frequencies for individuals is not known before exposure. Micronuclei harbouring whole chromosomes are primarily formed from failure of the mitotic spindle, kinetochore, or other parts of the mitotic apparatus or by damage to chromosomal sub-structures, alterations in cellular physiology, and mechanical disruption. An increased number of micronucleated cells is a biomarker of genotoxic effects and can reflect exposure to agents with clastogenic modes of action (chromosome breaking; DNA as target) or aneugenic ones (aneuplodigenic; effect on chromosome number; mostly non-DNA target) (Albertini, 2000). The advantage of the CBMN assay is its ability to detect both clastogenic and aneugenic events, leading to structural and numerical chromosomal aberrations, respectively (Kirsch-Volders et al., 2002; Mateuca et al., 2006). Micronuclei observed in cultured lymphocytes are believed to arise primarily *in vitro* from: 1) chromatid-type chromosomal aberrations formed during DNA replication on a damaged template, 2)

chromosome-type aberrations initiated before the mitosis and duplicated at replication, or 3) disturbances of mitotic apparatus leading to chromosome lagging. Micronuclei arising *in vivo*, inducible by both clastogenic and aneugenic mechanisms, can be scored in exfoliated epithelial cells (Salama et al., 1999) sampled, e.g., from buccal or nasal mucosa or urine, or in peripheral blood mononuclear cells e.g., isolated lymphocytes (Surrallés et al., 1996; Albertini, 2000). The CBMN assay is the preferred method for measuring micronuclei (MNi) in cultured human and/or mammalian cells because scoring is specifically restricted to once–divided cells. For scoring MNi uniform criteria should be used. Only MNi not exceeding 1/3 of the main nucleus diameter, clearly separable from the main nucleus and with distinct borders and of the same color as the nucleus, should be scored. In practice, 1000–2000 cells are often scored per subject in lymphocyte studies utilising the CBMN technique, while more cells (3000–5000 per subject) are evaluated in epithelial cells due to the lower baseline MNi frequency. In the CBMN method, only binucleate cells should be analysed for MNi; further divisions of a binucleate cell, usually resulting in cells with 3–4 nuclei, are highly irregular and show high MNi rates (Fenech et al., 2003).

The discovery of kinetochore-specific antibodies in the serum of scleroderma CREST (*Calcinosis, Raynaud's phenomenon, Esophageal dysmotility, Sclerodactyly and Telangiectasia*) patients (Moroy et al., 1980) has made it possible to determine the contents of micronuclei. Kinetochore immunofluorescence has been rapidly developed for the *in situ* detection of aneuploidy and chromosome breakage in human micronuclei (Degrassi & Tanzarella, 1988; Hennig et al., 1988; Eastmond & Tucker, 1989). Also, the FISH technique using chromosome-specific DNA probes has improved the detection and evaluation of structural chromosomal aberrations. The combination of the micronucleus assay with FISH using a DNA probe specific to the centromeric regions, or with antibodies that specifically stain kinetochore proteins, provides the methodology to distinguish between micronuclei containing either one or several whole chromosomes, which are positively labeled (centromere positive micronucleus), or acentric chromosome fragments, which are unlabeled due to the absence of centromere (centromere negative micronucleus) (Natarajan et al., 1996; Mateuca et al., 2006; Benameur et al., 2011). Except for cytogenetic damage measured by the number and distribution of micronuclei, according to the new criteria for micronuclei scoring, the CBMN assay also detects the nucleoplasmic bridges (NPBs), as well as nuclear buds (NBUDs). Current evidence suggests that NPBs derive from dicentric chromosomes which the centromeres have been pulled to the opposite poles of the cell during the anaphase stage, and are therefore indicative of the DNA mis-repair, chromosome rearrangement or telomere end-fusion. According to the new criteria applicable to the CBMN assay, NBUDs arise from the elimination of the amplified DNA and possibly from the elimination of the DNA-repair complexes, which therefore, may be considered a marker of gene amplification and altered gene dosage (Fenech, 2006; Fenech & Crott, 2002; Fenech et al., 2011; Garaj- Vrhovac et al., 2008; Lindberg et al., 2006; Thomas et al., 2003).

The significance of the CBMN assay lies in the fact that every cell in the system studied is scored cytologically for its viability status (necrosis, apoptosis), its mitotic status (mononucleated, metaphase, anaphase, binucleated, multinucleated) and its chromosomal instability or damage status (presence of MNi, NPBs, NBUDs and number of centromere probe signals amongst nuclei of binucleated cell if such molecular tools are used in combination with the assay). In this respect, the micronucleus assay has evolved into a comprehensive method employed in measuring chromosomal instability of the phenotype and altered cell viability and represents an effective tool to be used in research of cellular

and nuclear dysfunctions caused by *in vitro* or *in vivo* exposure to toxic substances (Fenech, 2006; Garaj- Vrhovac et al., 2008; Thomas & Fenech, 2011).

4. Genotoxicity of *p,p'*-DDT in human peripheral blood lymphocytes

4.1 Methodology

4.1.1 Chemicals

Chromosome kit P was from Euroclone, Milan, Italy; RPMI 1640, was from Invitrogen, Carlsbad, CA, USA; cytochalasin B, histopaque-1119, ethidium bromide, low melting point (LMP) and normal melting point (NMP) agaroses were from Sigma, St Louis, MO, USA; heparinised vacutainer tubes from Becton Dickinson, Franklin Lakes, NJ, USA; acridine orange form Heidelberg, Germany; Giemsa from Merk, Darmastad, Germany; All other reagents used were laboratory-grade chemicals from Kemika, Zagreb, Croatia. DDT was administered as *p,p'*-DDT (Sulpeco, Bellefonte, PA, USA) in final concentration of 0.025 mg/L at different time points.

4.1.2 Blood sampling and treatment

Whole blood samples were taken from a healthy female donor who had not been exposed to ionizing radiation, vaccinated or treated with drugs for a year before blood sampling. Whole venous blood was collected under sterile conditions in heparinised vacutainer tubes containing lithium heparin as anticoagulant. The comet assay and the micronucleus assay were conducted on whole blood cultivated at 37 °C in an atmosphere with 5% CO_2 (Heraeus Heracell 240 incubator, Langenselbold, Germany). The whole blood was treated with 0.025 mg/L *p,p'*-DDT for 1, 2, 4, 8, 24 and 48 h for the cytotoxicity assay and alkaline comet assay and 24 and 48 h for the CBMN assay. In each experiment, a non treated control was included.

4.1.3 Cell viability (cytotoxicity) assay

The indices of cell viability and necrosis were established by differential staining of human peripheral blood lymphocytes (HPBLs) with acridine orange and ethidium bromide using fluorescence microscopy (Duke & Cohen, 1992). Lymphocytes were isolated using a modified Ficoll-Histopaque centrifugation method (Singh, 2000). The slides were prepared using 200 μL of HPBLs and 2 μL of stain (acridine orange and ethidium bromide). The suspension mixed with dye was covered with a cover slip and analyzed under the epifluorescence microscope (Olympus AX 70, Tokyo, Japan) using a 60× objective and fluorescence filters of 515–560 nm. A total of 100 cells per repetition were examined. The nuclei of vital cells emitted a green fluorescence and necrotic cells emitted red fluorescence.

4.1.4 The alkaline comet (SCGE) assay

The alkaline comet assay was carried out as described by Singh et al. (1988). Briefly, after the exposure to *p,p'*-DDT, 5 μL of whole blood was mixed with 100 μL of 0.5% LMP agarose and added to fully frosted slides pre-coated with 0.6% NMP agarose. After solidifiying, the slides were covered with 0.5% LMP agarose, and the cells were lysed (2.5 M NaCl, 100 mM $EDTANa_2$, 10mM Tris, 1% sodium sarcosinate, 1% Triton X-100, 10% dimethyl sulfoxide, pH 10) overnight at 4°C. After the lysis the slides were placed into alkaline solution (300 mM NaOH, 1 mM $EDTANa_2$, pH 13) for 20 min at 4°C to allow DNA unwinding and subsequently electrophoresed for 20 min at 1 V/cm. Finally, the slides were neutralized in

0.4 M Tris buffer (pH 7.5) for 5 minutes 3 times, stained with EtBr (20 μg/mL) and analyzed at 250× magnification using an epifluorescence microscope (Zeiss, Göttingen, Germany) connected through camera to an image analysis system (Comet Assay II; Perceptive Instruments Ltd., Haverhill, Suffolk, UK). The tail length parameter was used to measure the level of DNA damage and a total of 100 randomly captured nuclei were examined from each slide. Tail length (i.e. the length of DNA migration) is related directly to the DNA fragment size and is presented in micrometers (μm). It was calculated from the centre of the nucleus.

4.1.5 Cytokinesis-blocked micronucleus (CBMN) assay

The micronucleus assay was performed in agreement with guidelines by Fenech & Morley, (1985). After the exposure to *p,p'*-DDT the whole blood (500 μL) was incubated in a Euroclone medium at 37 °C in an atmosphere of 5% CO_2 in air. Cytochalasin-B was added at a final concentration of 3 μg/mL 44 h after the culture was started. The cultures were harvested at 72 h. The lymphocytes were fixed in methanol-acetic acid solution (3:1), air-dried and stained with 5% Giemsa solution. All slides were randomised and coded prior to analysis. Binuclear lymphocytes were analyzed under a light microscope (Olympus CX41, Tokyo, Japan) at 400× magnification. Micronuclei, nucleoplasmic bridges and nuclear buds were counted in 1000 binucleated cells and were scored according to the HUMN project criteria published by Fenech et al. (2003).

4.1.6 Statistics

For the results of the comet assay measured after treatment with *p,p'*-DDT statistical evaluation was performed using Statistica 5.0 package (StaSoft, Tulsa, OK, USA). Multiple comparisons between groups were done by means of ANOVA on log-transformed data. Post hoc analyses of differences were done by using the Scheffé test. Differences in the frequency of micronuclei, nucleoplasmic bridges, and nuclear buds were assessed using the chi-square test. $P<0.05$ was considered significant.

4.2 Results

4.2.1 Vital staining using ethidium bromide and acridine orange

The viability of HPBLs exposed to aqueous *p,p'*-DDT (0.025 mg/L) for different lengths of time as determined by acridine orange and ethidium bromide staining, using fluorescence microscopy was not significantly affected (data not shown). Changes were determined according to the different staining of the nucleus (Figure 2).

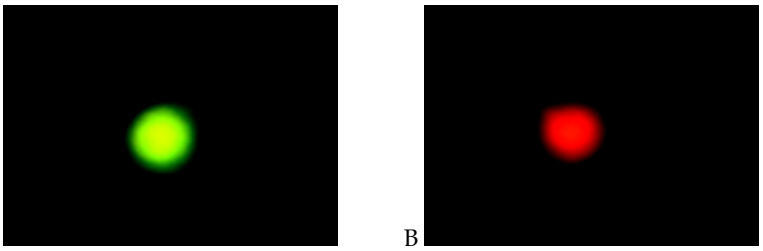


Fig. 2. Cell viability microphotographs represent viable lymphocyte from the un-exposed sample (A; green), and dead lymphocyte (B; red) from sample treated with aqueous solution of *p,p'*-DDT.

4.2.2 Induction of DNA strand breaks

The whole blood was exposed to aqueous solution of *p,p'*-DDT (0.025 mg/L) and the DNA damage in HPBLs was determined with the alkaline comet assay. Figure 3 represents different levels of DNA fragmentation between non-exposed sample and sample exposed to aqueous solution of *p,p'*-DDT. Statistically significant ($P<0.05$) increase in the amount of DNA strand breaks was observed after all exposure times to *p,p'*-DDT (Figure 4).

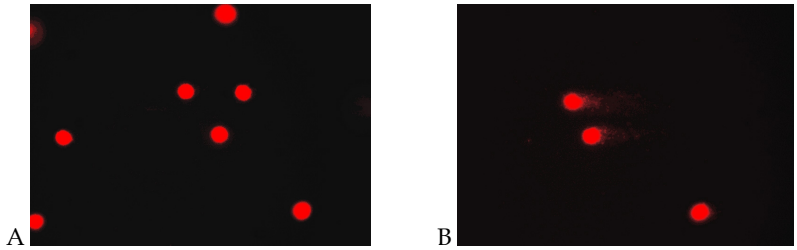


Fig. 3. Comet assay microphotographs represent undamaged lymphocytes from the un-exposed sample (A). Image (B) represents damaged lymphocytes that have comet appearance after the treatment with aqueous solution of *p,p'*-DDT.

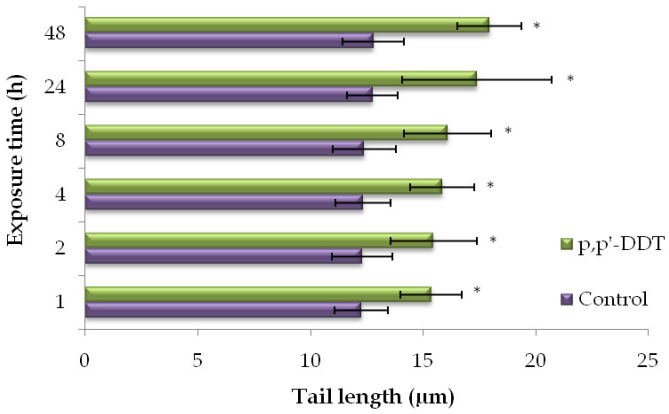


Fig. 4. Tail length (length of DNA migration) as comet assay parameter in human peripheral blood lymphocytes after exposure to low concentration of aqueous *p,p'*-DDT. * Statistically significant compared to corresponding control ($P<0.05$).

4.2.3 Induction of micronuclei, nucleoplasmic bridges and nuclear buds

The genotoxic activity of *p,p'*-DDT (0.025 mg/L) was further evaluated using the CBMN assay. Figure 5 represents binucleated lymphocytes from the non-exposed sample and samples exposed to aqueous solution of *p,p'*-DDT. Following *p,p'*-DDT treatment for 24 and 48 h, increase in the frequency of MNi was detected for both exposure times (Table 8). Additionally, statistically significant induction ($P<0.05$) of NBPs and NBUDs was also observed following *p,p'*-DDT treatment for 24 and 48 h (Table 9).

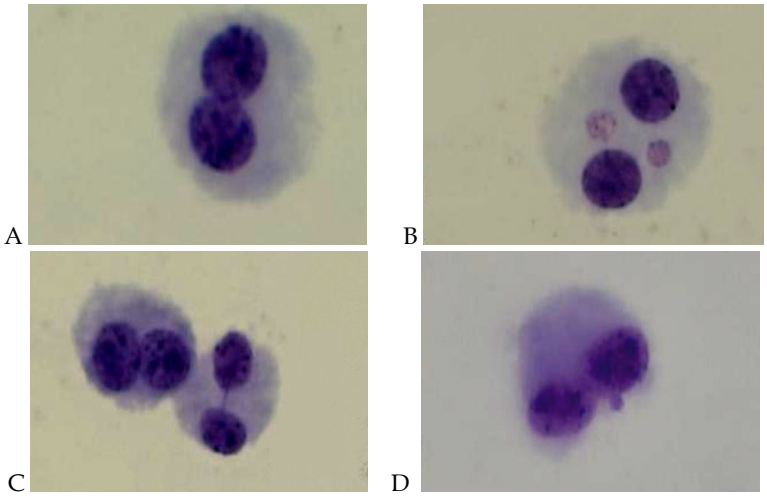


Fig. 5. Cytokinesis-block miconucleus assay microphotographs represent binucleated lymphocyte from the un-exposed sample (A). Image (B) represents binucleated lymphocyte with two micronuclei, image (C) binucleated lymphocyte with nucleoplasmic bridge (right), and image (D) binucleated lymphocyte with nucler bud after the treatment with aqueous solution of *p,p'*-DDT.

Exposure time (h)	Sample	1 MN	2 MNi	3 MNi	Total no. of MNi
24	Control	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	p,p'-DDT	28.50±13.44*	1.50±2.12	0.50±0.71	33.00±19.80*
48	Control	6.00±1.41	0.00±0.00	0.00±0.00	6.00±1.41
	p,p'-DDT	30.50±9.19*	1.50±2.12	0.50±0.71	35.00±15.56*

Table 8. Incidence of micronuclei (MNi) as cytokinesis-block micrunucleus assay parameter in human peripheral blood lymphocytes after exposure to low concentration of aqueous *p,p'*-DDT, * Statistically significant compared to corresponding control (P<0.05).

Exposure time (h)	Sample	Total no. of NPBs	Total no. of NBUDs
24	Control	0.00±0.00	0.00±0.00
	p,p'-DDT	10.50±0.71*	10.50±4.95*
48	Control	0.00±0.00	2.50±0.71
	p,p'-DDT	14.00±11.31*	20.50±3.54*

Table 9. Incidence of nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) as cytokinesis-block micrunucleus assay parameters in human peripheral blood lymphocytes after exposure to low concentration of aqueous *p,p'*-DDT, * Statistically significant compared to corresponding control (P<0.05).

5. Discussion

Due to uncontrolled use for several decades, many pesticides, among them DDT, probably the best known and the most useful insecticide in the world has damaged wild life and might have adverse effects on human health. Because of its current use in countries of the Third World, DDT still enters environment and in that way it can still represent health risk for human population, even in countries that have banned its use almost 40 years ago (ATSDR, 2002; Crinnion, 2009; Ecobichon, 2000, 2001; Eskenazi et al., 2009; Gajski et al., 2007; Turusov et al., 2002).

Large number of epidemiological studies regarding health risk of DDT confirmed that it represents major threat for wild life and human health (Aronson et al., 2010; Demers et al., 2000; Donato & Zani, 2010; Ecobichon, 1995; Martin et al., 2002; van Wendel de Joode et al., 2001; Wojtowicz et al., 2004, 2007; Woolcoot et al., 2001). Although tested in several genotoxicity studies on bacterial and animal models (Amer et al., 1996; Binneli et al., 2007, 2008a, 2008b; Canales-Aquirre, et al., 2011; Donnato et al., 1997a, 1997b; Galindo Reyes et al., 2002; Gauthier et al., 1999; Uppala et al., 2005) there is still necessity for conducting cytogenetic research regarding its genotoxicity employing sensitive methods to reveal the exact mechanisms of action of this chemical.

Combining different cytogenetic methods may play an important role in assessing genotoxic damage from different environmental chemical or physical agents. With these methods it is possible to evaluate the level of primary DNA damage or the dynamics of its repair even after short-term exposure to these agents (Garcia-Sagredo, 2008; Gajski & Garaj-Vrhovac, 2008). The comet assay is a sensitive method for measuring and analyzing DNA damage at the single cell level, and can be used both in *in vivo* and *in vitro* (Collins, 2004; Collins et al., 2008; Dušinská & Collins, 2008). The comet assay detects single and double stranded breaks at the level of DNA molecule, sites of incomplete repair, alkali labile sites, and DNA-DNA and DNA-protein cross-links (Piperakis, 2009). Furthermore, micronucleus assay can indicate cellular and nuclear dysfunction caused by *in vitro* or *in vivo* exposure to toxic substances. It is a reliable method for measuring chromosomal instability and altered cellular viability (Fenech, 2009; Fenech et al., 2003). It includes micronuclei, which are biomarkers of chromosome breakage and whole chromosome loss, nucleoplasmic bridges, which are biomarkers of DNA misrepair and telomere end-fusions, and nuclear buds, which are biomarkers of elimination of amplified DNA and DNA repair complexes (Fenech, 2007; Garaj-Vrhovac et al., 2008).

Considering the lack of data on the effect of DDT on the cellular genome, and taking into account its usage in some countries of the Third World and its environmental persistence, the aim of this study was to evaluate the genotoxic potential of a low concentration of aqueous *p,p'*-DDT upon *in vitro* exposure of HPBLs of different duration, by using alkaline comet assay and CBMN assay. Our results showed that exposure of HPBLs to aqueous *p,p'*-DDT increased DNA damage in time dependent fashion as measured by the comet assay. In addition, CBMN assay parameters revealed a wider scale of chromosomal alterations after *p,p'*-DDT treatment.

Cytogenetic studies of DDT are mainly based on *in vitro* research on the animal models and its genotoxicity was evaluated in a variety of test systems. Results obtained by studying cytogenetic effects of DDT on DNA of shrimp larvae (*Litopenaeus stylirostris*) indicated that DDT causes DNA adducts and/or breaks (Galindo Reyes et al., 2002). DDT and its

metabolites DDE and DDD showed a clear genotoxic effect on haemocytes of zebra mussel (*Dreissena polymorpha*) specimens in different concentrations that have been found in several aquatic ecosystems worldwide, with a greater genotoxic potential of the DDE in respect to the other two chemicals (Binneli et al., 2008a, 2008b). DDT has also the ability to induce chromosomal aberrations in mouse spleen indicating its genotoxicity (Amer at al., 1996). In addition, DDT was genotoxic towards lymphocytes and mammary epithelial cells of female rats showing an increase in lipid peroxidation, the outcome of the growth level of free oxygen radicals, which lead to an oxidative stress (Canales-Aquirre et al., 2011). DDT also induces cellular and chromosomal alterations in the rat mammary gland, which is consistent with the hypothesis that it can induce early events in mammary carcinogenesis (Uppala et al., 2005). Additionally, beluga whales (*Delphinapterus leucas*) inhabiting the St. Lawrence estuary are highly contaminated with environmental pollutants including DDT which can induced significant increases of micronucleated cells in skin fibroblasts of an Arctic beluga whale (Gauthier et al., 1999).

Regarding human test system, the cytogenetic effect of DDT was investigated both *in vitro* and *in vivo*. *In vitro*, certain DDT concentrations have the effects on human leukocyte functions (Lee et al., 1979), are causing chromosomal aberrations (Lessa et al., 1976), DNA strand breaks (Yáñez et al., 2004), and apoptosis induction which is preceded by an increase in the levels of reactive oxygen species (Pérez-Maldonado et al., 2004, 2005). *In vivo*, DDT is able to induce chromatid lesions (Rabello et al., 1975), increase in chromosomal aberrations and sister chromatid exchanges (Rupa et al., 1989, 1991), DNA strand breaks (Yáñez et al., 2004; Pérez-Maldonado et al., 2006), apoptosis (Pérez-Maldonado et al., 2004) as well as cell cycle delay and decrease in mitotic index (Rupa et al., 1991).

Before 1973 when it was banned, DDT entered the air, water and soil during its production and use as an insecticide. DDT is present at many waste sites and from these sites it might continue to contaminate the environment. DDT still enters the environment because of its current use in other areas of the world. DDT may be released into atmosphere in countries where it is still manufactured and used; it can also enter the air by evaporation from contaminated water and soil and than it can be deposited on land or surface water. This cycle of evaporation and deposition may be repeated many times and as a result, DDT can be carried long distances in the atmosphere (ATSDR, 2002; Crinnion, 2009; Donato & Zani, 2010; Gajski et al., 2007; Torres-Sánchez & López-Carrillo 2007). These chemicals have been found in bogs, snow and animals even in the Artic and Antarctic regions, far from where they were ever used. DDT can last in the soil for a very long time, potentially for hundreds of years. Most DDT breaks down slowly into DDE and DDD, generally by the action of microorganisms and can be deposited in other places like in the surface layers of soil; it may get into rivers and lakes in runoff or get into groundwater. In surface waters, DDT will bind to particles in the water, settle and be deposited in the sediment. DDT is than taken up by small organisms and fish in the water. It accumulates to high levels in adipose tissue of fish and marine mammals, reaching levels many thousands of times higher than in water. DDT can also be absorbed by some plants and by the animals that can directly impact human population and like that represent a major health threat (ATSDR, 2002; Beard, 2006; Gajski et al., 2007; Gauthier et al., 1999).

All of these findings suggests that DDT is still present not only in poorly developed countries of the Third World but it can still be found in other countries that have banned its use almost 40 years ago due to its stability and long persistence in the environment.

6. Conclusion

Significant levels of DDT and its metabolites can still be found in biological samples of serum, adipose tissue and maternal milk of populations that are not occupationally exposed. People are usually exposed to DDT through food, inhalation or dermal contact. Also, there are evidences on damages to the health, specially related to the reproductive area, and more recently damages at cellular level, as well as, alteration in the psychomotor development of children exposed in uterus. Although there are studies dealing with adverse effects of pesticide exposure there is still great need for elucidating the exact mechanism and health consequences related to DDT exposure and its metabolites. Our data in conjunction with other available data regarding pesticide genotoxicity have identified that DDT induces DNA strand breaks in human peripheral blood lymphocytes *in vitro* as well *in vivo*. In our study, this effect was noted even after the treatment with very low concentration of aqueous DDT. These results also confirms previous findings that DDT induces alterations of the ultrastructure of cells and DNA damage by causing single strand breakage and adducts in DNA molecule. Present study also confirms that combinations of sensitive techniques like alkaline comet assay and cytokinesys-block micronucleus assay are useful for the assessment of cellular and DNA alterations after exposure to mutagens and carcinogens from the environment. Results obtained in this research indicate the need for further environmental and food monitoring, and cytogenetic research using sensitive methods in detection of primary genome damage after exposure to DDT to establish the impact of such chemicals on human genome and health.

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8. References

Agency for Toxic Substances and Disease Service. (2002). *Toxicological profile for DDT, DDE and DDD*, U.S. Department of Health and Human Services, Public Health Service, ATSDR, Atlanta, USA

Albertini, R.J.; Anderson, D.; Douglas, G.R.; Hagmar, L.; Hemminki, K.; Merlo, F.; Natarajan, A.T.; Norppa, H.; Shuker, D.E.G.; Tice, R.; Waters, M.D. & Aitio, A. (2000). IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. *Mutation Research*, Vol.463, No.2, pp. 111–172, ISSN 1383-5742

Amer, S.M.; Fahmy, M.A. & Donya, S.M. (1996). Cytogenetic effect of some insecticides in mouse spleen. *Journal of Applied Toxicology*, Vol.16, No.1, pp. 1-3, 1099-1263, ISSN 0260-437X

Angelis, K.J.; Dušinská, M. & Collins, A.R. (1999). Single cell gel electrophoresis: detection of DNA damage at different levels of sensitivity. *Electrophoresis,* Vol.20, No.10, pp. 2133-2138, ISSN 0173–0835

Aronson, K.J.; Wilson, J.W.; Hamel, M.; Diarsvitri, W.; Fan, W.; Woolcott, C.; Heaton, J.P.; Nickel, J.C.; Macneily, A. & Morales, A. (2010). Plasma organochlorine levels and

prostate cancer risk. *Journal of Exposure Science and Environmental Epidemiology*, Vol.20, No.5, pp. 434-445, ISSN 1559-0631

Arutyunyan, R.; Rapp, A.; Greulich, K.O.; Hovhannisyan, G.; Haroutiunian, S. & Gebhart, E. (2005). Fragility of telomeres after bleomycin and cisplatin combined treatment measured in human leukocytes with the Comet–FISH technique. *Experimental Oncology,* Vol.27, No.1, pp. 38-42, ISSN 1812–9269

Ashman, C.R. & Davidson, R.L. (1981). Bromodeoxyuridine mutagenesis in mammalian cells is related to deoxyribonucleotide pool imbalance, *Molecular and cellular biology*, Vol.1, No.3, pp.254–260, ISSN 0270-7306

Aubé, M., Larochelle, C. & Ayotte, P. (2008). 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (*p,p*'-DDE) disrupts the estrogen-androgen balance regulating the growth of hormone-dependent breast cancer cells. *Breast Cancer Research*, Vol.10, No.1, R16, ISSN 1465-5411

Bakić, J. (2011) Seven centuries of the prevention of spreading of the infectious diseases into Croatia - Overview of the 60th anniversary of establishment of modern pest control in Croatia. *Hrvatski Časopis za Javno Zdravstvo*, Vol.7, No.25, ISSN 1845-3082

Bartsch, H. (1996). DNA adducts in human carcinogenesis: Etiological relevance and structure-activity relationship. *Mutation Research,* Vol.340, No.2, pp. 67-79, ISSN 1383-5742

Beard, J, & Australian Rural Health Research Collaboration. (2006). DDT and human health. *Science of the Total Environment*, Vol.355, No.1-3, pp. 78-89, ISSN 0048-9697

Benameur, L.; Orsière, T.; Rose, J. & Botta, A. (2011). Detection of environmental clastogens and aneugens in human fibroblasts by cytokinesis-blocked micronucleus assay associated with imunofluorescent staining of CENP-A in micronuclei. *Chemosphere,* Vol.84, No.5, pp, 676-680, ISSN 0045-6535

Binelli, A.; Riva, C.; Cogni, D. & Provini, A. (2008a). Genotoxic effects of p,p'-DDT (1,1,1-trichloro-2,2-bis-(chlorophenyl)ethane) and its metabolites in Zebra mussel (D. polymorpha) by SCGE assay and micronucleus test. *Environmental and Molecular Mutagenesis*, Vol.49, No.5, pp. 406-415, ISSN 1098-2280

Binelli, A.; Riva, C.; Cogni, D. & Provini A. (2008b). Assessment of the genotoxic potential of benzo(a)pyrene and pp'-dichlorodiphenyldichloroethylene in Zebra mussel (Dreissena polymorpha). *Mutation Research*, Vol.649, No.1-2, pp. 135-145, ISSN 1383-5718

Binelli, A.; Riva, C. & Provini, A. (2007). Biomarkers in Zebra mussel for monitoring and quality assessment of Lake Maggiore (Italy). *Biomarkers*, Vol.12, No.4, pp. 349-368, ISSN 1354-750X

Boiteux, S. & Radicella, P. (2000). The human OGG1 gene: structure, functions, and its implication in the process of carcinogenesis. *Archives of Biochemistry and Biophysics,* Vol. 377, No. 1, pp. 1– 8, ISSN 0003–9861

Boller, K. & Schmid, W. (1970). Chemical mutagenesis in mammals. The Chinese hamster bone marrow as an in vivo test system. Hematological findings after treatment with trenimon. *Humangenetik,* Vol.11, No.1, pp. 35–54, ISSN 0018–7348

Bolognesi, C. (2003). Genotoxicity of pesticides: a review of human biomonitoring studies. *Mutation Research*, Vol.543, No.3, pp. 251–272, ISSN 1383-5742

Bošnir, J.; Puntarić, D.; Smit, Z.; Klarić, M.; Grgić, M. & Kosanović, L.M. (2007). Organochlorine pesticides in freshwater fish from the Zagreb area. *Arhiv za Higijenu Rada i Toksikologiju,* Vol.58, No.2, pp. 187-193, ISSN 0004-1254

Bošnir, J.; Novoselac, V.; Puntarić, D.; Klarić, I.; Miškulin, M. (2010). Organochlorine pesticide residues in cow's milk from Karlovac County, Croatia. *Acta Alimentaria.* Vol.39, No.3, pp. 317-326, ISSN 0139-3006

Brooks, G.T. (1986). Insecticide metabolism and selective toxicity. *Xenobiotica*, Vol.16, No.10-11, pp. 989-1002, ISSN 0049-8254

Buckton, K.E.; Court Brown, W.M. & Smith, P.G. (1967). Lymphocyte survival in men treated with X-rays for ankylosing spondylitis. *Nature*, Vol.241, No.5087, pp. 470-473, ISSN 0028-0836

Bulut, S.; Akkaya, L.; Gok, V.; & Konuk, M. (2010). Organochlorine pesticide residues in butter and kaymak in afyonkarahisar, Turkey. *Journal of Animal and Veterinary Advances*, Vol.9, No.22, pp. 2797-2801, ISSN 1680-5593

Canales-Aguirre, A.; Padilla-Camberos, E.; Gómez-Pinedo, U.; Salado-Ponce, H.; Feria-Velasco, A. & De Celis, R. (2011). Genotoxic effect of chronic exposure to DDT on lymphocytes, oral mucosa and breast cells of female rats. *International Journal of Environmental Research and Public Health*, Vol.8, No.2, pp. 540-553, ISSN 1661-7827

Carson, R. (2002). *Silent Spring*, Mariner Books, ISBN 0618249060, New York, USA

Charlwood, D. (2003). Did Herodotus describe the first airborne use of mosquito repellents. *Trends in Parasitology*, Vol. 19, No. 12, pp. 555-556, 1471-4922

Collins, A.R. (2004). The comet assay for DNA damage and repair: principles, applications, and limitations. *Molecular Biotechnology*, Vol.26, No.3, pp. 249-261, ISSN 1073–6085

Collins, A.R. (2009). Investigating oxidative DNA damage and its repair using the comet assay. *Mutation Research*, Vol.681, No.1, pp. 24-32, ISSN 1383-5742

Collins, A.R.; Dobson, V.L.; Dušinská, M.; Kennedy, G. & Stetina, R. (1997). The comet assay: what can it really tell us? *Mutation Research,* Vol.375, No.2, pp. 183-193, ISSN 0027-5107

Collins, A.R.; Oscoz, A.A.; Brunborg, G.; Gaivão, I.; Giovannelli, L.; Kruszewski, M.; Smith, C.C. & Stetina, R. (2008). The comet assay: topical issues. *Mutagenesis*, Vol.23, No.3, pp. 143-151, ISSN 1464-3804

Countryman, P.I. & Heddle, J.A. (1976). The production of micronuclei from chromosome aberrations in irradiated cultures of human lymphocytes. *Mutation Research*, Vol.41, No.2-3, pp. 321–332, ISSN 0027–5107

Covaci, A.; Gheorghe, A. & Schepens, P. (2004). Distribution of organochlorine pesticides, polychlorinated biphenyls and a-HCH enantiomers in pork tissues. *Chemosphere*, Vol.56, No.8, pp. 757–766, ISSN 0045-6535

Crinnion, W.J. (2009). Chlorinated pesticides: threats to health and importance of detection. *Alternative Medicine Review*, Vol. 14, No. 4, pp. 347-359, ISSN 1089-5159.

Crosby, D.G. (1982). Pesticides as environmental mutagens, In: *Genetic Toxicology: An agricultural Perspective,* R.A. Fleck & A. Hollander, (Eds.), 201-218, Plenum Press, ISBN 9780306411359, New York, USA

Dawood, A.W.A.; Abd El-Maaboud, R. M.; Helal, M.A.; Mohamed, S.A. & Waleed, H.A. (2004). Detection of organochlorine pesticide residues in samples of cow milk

collected from Sohag and Qena overnorates. *Assiut University Bulletin for Environmental Researches*, Vol.7, No.2, pp. 105-116, ISSN 1110-6107

Degrassi, F. & Tanzarella, C. (1988). Immunofluorescent staining of kinetochores in micronuclei: a new assay for the detection of aneuploidy. *Mutation Research*, Vol.203, No.5, pp. 339–345, ISSN 0165-7992

Demers, A.; Ayotte, P.; Brisson, J.; Dodin, S.; Robert, J. & Dewailly, E. (2000). Risk and aggressiveness of breast cancer in relation to plasma organochlorine concentrations. *Cancer Epidemiology, Biomarkers and Prevention*, Vol.9, No.2, pp. 161-166, ISSN 1055-9965

Doetsch, P.W.; Henner, W.D.; Cunningham, R.P.; Toney, J.H. & Helland, D.E. (1987). A highly conserved endonuclease activity present in *Escherichia coli*, bovine, and human cells recognizes oxidative DNA damage at sites of pyrimidines. *Molecular and Cellular Biology*, Vol.7, No.1, pp. 26–32, ISSN 0270-7306

Donato, M.M.; Jurado, A.S.; Antunes-Madeira, M.C. & Madeira, V.M. (1997a). Bacillus stearothermophilus as a model to evaluate membrane toxicity of a lipophilic environmental pollutant (DDT). *Archives of Environmental Contamination and Toxicology*, Vol.33, No.2, pp. 109-116, ISSN 0090-4341

Donato, M.M.; Jurado, A.S.; Antunes-Madeira, M.C. & Madeira, V.M. (1997b) Effects of a lipophilic environmental pollutant (DDT) on the phospholipid and fatty acid contents of Bacillus stearothermophilus. *Archives of Environmental Contamination and Toxicology*, Vol.33, No.4, pp. 341-349, ISSN 0090-4341

Donato, F. & Zani, C. (2010). Chronic exposure to organochlorine compounds and health effects in adults: diabetes and thyroid diseases. *Annali di Igiene: Medicina Preventiva e di Comunita*, Vol.22, No.3, pp. 185-198, ISSN 1120-9135

Drevenkar, V. & Fingler, S. (2000). Persistent organochlorine compounds in Croatia. *Arhiv za Higijenu Rada i Toksikologiju*, Vol.51, No.Suppl, pp. 59–73, ISSN 0004-1254

Drevenkar, V.; Fingler, S. & Fröbe, Z. (1994). Some organochlorine pollutants in the water environment and their influence on drinking water quality, In: *Chemical Safety, International Reference Manual,* Richardson, M.L. (Ed.), 297-310, Wiley-VCH, ISBN 9783527286300, Weinheim, Germany

Dugački, V. (2005). Dr. Rudolf Battara operation in Nin in 1902, the first systematic battle attempt against malaria in Croatia. *Medica Jadertina*, Vol.35, No.Suppl, pp. 33-40, ISSN 0351-0093

Duke, R.C. & Cohen, J.J. (1992). Morphological and biochemical assays of apoptosis, In: *Current Protocols in Immunology*, J.E. Coligan & A.M. Kruisbeal, (Eds.), 1-3, John Willey & Sons, ISBN 9780471522768, New York, USA

Dušinská, M. & Collins, A. (1996). Detection of oxidised purines and UV-induced photoproducts in DNA of single cells, by inclusion of lesion-specific enzymes in the comet assay. *Alternatives to Laboratory Animals*, Vol.24, No.3, pp. 405–411, ISSN 0261-1929

Dušinská, M. & Collins, A.R. (2008). The comet assay in human biomonitoring: gene-environment interactions. *Mutagenesis*, Vol.23, No.3, pp. 191-205, ISSN 1464-3804

Eastmond, D. A. & Tucker, J. D. (1989). Identification of aneuploidyinducing agents using cytokinesis-blocked human lymphocytes and an anti-kinetochore antibody. *Environmental and Molecular Mutagenesis*, Vol.13, No.1, pp. 34-43, ISSN 0893-6692

Ecobichon, D.J. (1995). Toxic effects of pesticides, In: Casarett and Doull's toxicology: the Basic Science of Poisons, C.D. Klaassen, M.O. Amdur & J. Doull, (Eds.), 643-689, Macmillan Co., ISBN 0071054766, New York, USA

Ecobichon, D.J. (2000). Our changing perspectives on benefits and risks of pesticides: a historical overview. *Neurotoxicology*, Vol.21, No.1-2, pp. 211-218, ISSN 0161-813X

Ecobichon, D.J. (2001). Pesticide use in developing countries. *Toxicology*, Vol.160, No.1-3, pp. 27-33, ISSN 0300-483X

El-Zein, R.A.; Monroy, C.M.; Cortes, A.; Spitz, M.R.; Greisinger, A. & Etzel, C.J. (2010). Rapid method for determination of DNA repair capacity in human peripheral blood lymphocytes amongst smokers. *Bio Med Central Cancer*, Vol.10, No.1, pp. 439, ISSN 1471-2407

Eskenazi, B.; Chevrier, J.; Rosas, L.G.; 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, pp. 1359-1367, ISSN 0091-6765

Fenech, M. (1991). Optimisation of micronucleus assays for biological dosimetry. *Progress in Clinical and Biological Research*, Vol.372, pp. 373-386, ISSN 0361-7742

Fenech, M. (2000) The in vitro micronucleus technique. *Mutation Research*, Vol.455, No.1-2, pp. 81-95, ISSN 0027-5107

Fenech, M. (2006). Cytokinesis-block micronucleus assay evolves into a "cytome" assay of chromosomal instability, mitotic dysfunction and cell death. *Mutation Research*, Vol.600, No.1-2, pp. 58-66, ISSN 0027-5107

Fenech, M. (2007). Cytokinesis-block micronucleus cytome assay. *Nature Protocols*, Vol.2, No.5, pp. 1084-1104, ISSN 1750-2779

Fenech, M. (2009). A lifetime passion for micronucleus cytome assays - reflections from Down Under. *Mutation Research*, Vol.681, No.2-3, pp. 111-117, ISSN 1383-5742

Fenech, M.; Perepetskaya, G. & Mikhalevic, L. (1997). A more comprehensive application of the micronucleus technique for biomonitoring of genetic damage rates in human populations. Experiences from the Chernobyl catastrophe, *Environmental and Molecular Mutagenesis,* Vol.30, No.2, pp.112–118, ISSN 0893-6692

Fenech. M.; Chang, W.P.; Kirsch-Volders, M.; Holland, N.; Bonassi, S.; Zeiger, E. & HUman MicronNucleus project. (2003). HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. *Mutation Research*, Vol.534, No.1-2, pp. 65-75, ISSN 1383-5718

Fenech, M. & Crott, J.W. (2002). Micronuclei, nucleoplasmic bridges and nuclear buds induced in folic acid deficient human lymphocytes-evidence for breakage-fusion-bridge cycles in the cytokinesis-block micronucleus assay. *Mutation Research,* Vol.504, No.1-2, pp. 131-136, ISSN 0027-5107

Fenech, M.; Kirsch-Volders, M.; Natarajan, A.T.; Surralles, J.; Crott, J.W.; Parry, J.; Norppa, H.; Eastmond, D.A.; Tucker, J.D. & Thomas, P. (2011). Molecular mechanisms of

micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. *Mutagenesis*, Vol.26, No.1, pp. 125-132, ISSN 0267-8357

Fenech, M. & Morley, A. A. (1985). Measurement of micronuclei in lymphocytes. *Mutation Research*, Vol.147, No.1-2, pp. 29-36, ISSN 0027-5107

Fenech, M. & Morley, A.A. (1986). Cytokinesis-block micronucleus method in human lymphocytes: effect of in vivo ageing and low dose X- irradiation. *Mutation Research*, Vol.161, No.2, pp. 193–198, ISSN 0027–5107

Fenech, M. & Morley, A.A. (1989). Kinetochore detection in micronuclei: an alternative method for measuring chromosome loss. *Mutagenesis*, Vol.4, No.2, pp. 98-104, ISSN 0267-8357

Fernández, J.L.; Vázquez-Gundín, F.; Rivero, M.T.; Genescá, A.; Gosálvez, J. & Goyanes, V. (2001). DBD-fish on neutral comets: simultaneous analysis of DNA single- and double-strand breaks in individual cells. *Experimental Cell Research*, Vol.270, No.1, pp. 102-109, ISSN 0014-4827

Fingler, S.; Drevenkar, V.; Tkalčević, B. & Šmit, Z. (1992). Levels of polychlorinated biphenyls, organochlorine pesticides, and chlorophenols in the Kupa river water and in drinking waters from different areas in Croatia, *Bulletin of Environmental Contamination and Toxicology*, Vol. 49, No. 6, pp. 805-812 ISSN 1432-0800

Food and Agriculture Organization of United Nations. (2005). *International Code of Conduct on the Distribution and Use of Pesticides*, FAO, ISBN 9251054118, Rome, Italy

Fučić, A.; Markučič, D.; Mijić, A. & Jazbec, A.M. (2000). Estimation of genome damage after exposure to ionising radiation and ultrasound used in industry. *Environmental and molecular mutagenesis*, Vol.36, No.1, pp. 47–51, ISSN 0893-6692

Gajski, G. & Garaj-Vrhovac, V. Application of cytogenetic endpoints and comet assay on human lymphocytes treated with atorvastatin in vitro. *Journal of Environmental Science and Health Part A Toxic/Hazardous Substances & Environmental Engineering*, Vo.43, No.1, pp. 78-85, ISSN 1093-4529

Gajski, G.; Garaj-Vrhovac, V. & Oreščanin V. (2008). Cytogenetic status and oxidative DNA-damage induced by atorvastatin in human peripheral blood lymphocytes: standard and Fpg-modified comet assay. *Toxicology and Applied Pharmacology*, Vol.231, No.1, pp. 85-93, ISSN 0041-008X

Gajski, G.; Ravlić, S.; Capuder, Ž. & Garaj-Vrhovac, V. (2007). Use of sensitive methods for detection of DNA damage on human lymphocytes exposed to p,p'-DDT: Comet assay and new criteria for scoring micronucleus test. *Journal of Environmental Science and Health Part B*, Vol.42, No.6, pp. 607-613, ISSN 0360-1234

Galassi, S.; Bettinetti, R.; Neri, M.C.; Falandysz, J.; Kotecka, W.; King, I.; Lo, S.; Klingmueller, D. & Schulte-Oehlmann, U. (2008). *p,p'*-DDE contamination of the blood and diet in central European populations. *Science of the Total Environment,* Vol. 390, No.1, pp. 45-52, ISSN 0048-9697

Galindo Reyes, J.G.; Leyva, N.R.; Millan, O.A. & Lazcano, G.A. (2002). Effects of pesticides on DNA and protein of shrimp larvae Litopenaeus stylirostris of the California Gulf. *Ecotoxicology and Environmental Safety*, Vol.53, No.2, pp. 191-195, ISSN 0147-6513

Garaj-Vrhovac, V.; Gajski, G. & Ravlić, S. (2008). Efficacy of HUMN criteria for scoring the micronucleus assay in human lymphocytes exposed to a low concentration of p,p'-DDT. *Brazilian Journal of Medical and Biological Research*, Vol.41, No.6, pp. 473-476, ISSN 1414-431X

Garcia-Sagredo, J.M. (2008). Fifty years of cytogenetics: a parallel view of the evolution of cytogenetics and genotoxicology. *Biochimica et Biophysica Acta*, Vol.1779, No.6-7, pp. 363-375, ISSN 0006-3002

Gauthier, J.M.; Dubeau, H. & Rassart, E. (1999). Induction of micronuclei in vitro by organochlorine compounds in beluga whale skin fibroblasts. *Mutation Research*, Vol.439, No.1, pp. 87-95, ISSN 1383-5718

Glynn, A.; Lignell, S.; Darnerud, P.O.; Aune, M.; Ankarberg, E.H.; Bergdahl, I.A.; Barregård, L. & Bensryd, I. (2011). Regional differences in levels of chlorinated an brominated pollutants in mother's milk from primiparous women in Sweden. *Environment International, Vol.* 37, No. 1, pp. 71-79, ISSN 0160-4120

Gold, B. & Brunk, G. (1982). Metabolism of 1,1,1-trichloro-2,2-bis(p-chlorophenyl)-ethane and 1,1-dichloro-2,2-bis(p-chlorophenyl)ethane in the mouse. *Chemico-Biological Interactions*, Vol.41, No.3, pp. 327-339, ISSN 0009-2797

Gold, B. & Brunk, G. (1983). Metabolism of 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT), 1,1-dichloro-2,2-bis(p-chlorophenyl)ethane, and 1-chloro-2,2-bis(p-chlorophenyl)ethene in the hamster. *Cancer Research*, Vol.43, No.6, pp. 2644-2647, ISSN 0008-5472

Hagmar, L.; Brogger, A.; Hansteen, I.L.; Heim, S.; Hogstedt, B.; Knudsen, L.; Lambert, B.; Linnainmaa, K.; Mitelman, F.; Nordenson, I.; Reuterwall, C.; Salomaa, S.I.; Skerfving, S. & Sorsa, M. (1994). Cancer risk in human predicted by increased levels of chromosomal aberrations in lymphocytes: Nordic Study Group on the Health Risk of Chromosome Damage. *Cancer research*, Vol.54, No.11, pp. 2919–2922, ISSN 0008-5472

Hagmar, L.; Bonassi, S.; Stromberg, U.; Brogger, A.; Knudsen, L.; Norppa, H. & Reuterwall, C. (1998). The European Study Group on Cytogenetic Biomarkers and Health, Chromosomal aberrations in lymphocytes predict human cancer. A report from the European Study Group on Cytogenetic Biomarkers and Health (ESCH), *Cancer Research*, Vol.58, No.18, pp. 4117–4121, ISSN 0008-5472

Hamel, D. (2003). Inventarizacija perzistentnih organskih onečišćavala – pesticidi, Available from http://www.cro-cpc.hr/projekti/pops/Pest_Izvjestaj.pdf

Hansen, W.K. & Kelley, M.R. (2000). Review of mammalian DNA repair and translational implications. *The Journal of Pharmacology and Experimental Therapeutics*, Vol.295, No.1, pp. 1-9, ISSN 0022-3565

Harreus, U.A.; Kleinsasser, N.H.; Zieger, S.; Wallner, B.; Reiter, M.; Schuller, P. & Berghaus, A. (2004). Sensitivity to DNA-damage induction and chromosomal alterations in mucosa cells from patients with and without cancer of the oropharynx detected by a combination of Comet assay and fluorescence in situ hybridization. *Mutation Research*, Vol.563, No.2, pp. 131-138, ISSN 1383-5718

Hartmann, A.; Fender, H. & Speit, G. (1998). Comparative biomonitoring study of workers at a waste disposal site using cytogenetic tests and the Comet (Single-Cell Gel)

assay. *Environmental and Molecular Mutagenesis,* Vol.32, No.1, pp. 17-24, ISSN 0893-6692

Heck, M.C.; Sifuentes dos Santos, J.; Bogusz Junior, S.; Costabeber, I & Emanuelli, T. (2006). Estimation of children exposure to organochlorine compounds through milk in Rio Grande do Sul, Brazil. *Food Chemistry,* Vol.102, No.1, pp. 288-294, ISSN 0308-8146

Heddle, J.A. (1973). A rapid in vivo test for chromosome damage. *Mutation Research,* Vol.18, No.2, pp. 187–190, ISSN 0027-5107

Hennig, U.G.G.; Rudd, N.L. & Hoar, D.I. (1988). Kinetochore immunofluorescence in micronuclei: a rapid method for the in situ detection of aneuploidy and chromosome breakage in human fibroblasts. *Mutation Research*, Vol.203, No.6, pp. 405-414, ISSN 0165-7992

Horváthová, E.; Dusinská, M.; Shaposhnikov, S. & Collins, A.R. (2004). DNA damage and repair measured in different genomic regions using the comet assay with fluorescent in situ hybridization. *Mutagenesis,* Vol.19, No.4, pp. 269-276, ISSN 0267–8357

International Agency for Research on Cancer. (2011). *Agents reviewed by the IARC monographs*, Volumes 1-101, IARC, Lyon, France

International standard HRN EN ISO 6468. (2002). *Water quality-Determination of certain organochlorine insecticides, policlorinated biphenyls and chlorbenzenes-Gas chromatographic method after liqid-liquid extraction*

International standard ISO 10382. (2002). *Soil quality: Determination of organochlorine pesticides and polychlorinated biphenyls; Gaschromatographic method with electron capture detection*

Ji, B.T.; Silverman, D.T. & Stewart, P.A.; Blair, A.; Swanson, G.M.; Greenberg, R.S.; Hayes, R.B.; Brown, L.M.; Lillemoe, K.D.; Schoenberg, J.B.; Pottern, L.M.; Schwartz, A.G. & Hoover, R.N. (2001). Occupational exposure to pesticides and pancreatic cancer. *American Journal of Industrial Medicine*, Vol.39, No.1, pp. 92–99, ISSN 0271-3586

Johansen, P.; Muir, D.; Asmund, G. & Riget, F. (2004). Human exposure to contaminants in the traditional Greenland diet. *Science of the Total Environment*, Vol.331, No. 1-3, pp. 189–206, ISSN 0048-9697

Kamrin, M.A. (2000). *Pesticide Profiles, Toxicity, Environmental Impact, and Fate*, Lewis Publisher, ISBN 0849321794, New York, USA

Kirsch-Volders, M. & Fenech, M. (2001). Inclusion of micronuclei in non-divided mononuclear lymphocytes and necrosis/apoptosis may provide a more comprehensive cytokinesis block micronucleus assay for biomonitoring purposes. *Mutagenesis*, Vol.16, No.1, pp. 51-58, ISSN 0267-8357

Kirsch-Volders, M.; Sofuni, T.; Aardema, M.; Albertini, S.; Eastmond, D.; Fenech, M.; Ishidate, M.Jr.; Lorge, E.; Norppa, H.; Surrallés, J.; von der Hude, W. & Wakata, A. (2000). Report from the In Vitro Micronucleus Assay Working Group. *Environmental and Molecular Mutagenesis*, Vol.35, No.3, pp. 167-172, ISSN 0893-6692

Kirsch-Volders, M.; Vanhauwaert, A.; De Boeck, M. & Decordier, I. (2002). Importance of detecting numerical versus structural chromosome aberrations. *Mutation Research*, Vol.504, No.1-2, pp. 137–148, ISSN 0027-5107

Kodba, Z.C. & Voncina, D.B. (2007). A rapid method for the determination of organochlorine, pyrethroid pesticides and polychlorobiphenyls in fatty foods using

GC with electron capture detection. *Chromatographia*, Vol.66, No.7-8, pp. 619–624, ISSN 0009-5893

Krauthacker, B. & Reiner, E. (2001). Organochlorine compounds in human milk and food of animal origin in samples from Croatia. *Arhiv za Higijenu Rada i Toksikologiju*, Vol.52, Vol.2, pp. 217–227, ISSN 0004-154

Lander, B.F.; Knudsen, L.E.; Gamborg, M.O.; Jarventaus, H. & Norppa, H. (2000). Chromosome aberrations in pesticide-exposed greenhouse workers. *Scandinavian Journal of Work, Environment & Health*, Vol. 26, No.5, pp. 436–442, ISSN 0355-3140

Lee, T.P.; Moscati, R. & Park, B.H. (1979). Effects of pesticides on human leukocyte functions. *Research Communication in Chemical and Pathological Pharmacology*, Vol.23, No.3, pp. 597-609, ISSN 0034-5164.

Lessa, J.M.; Beçak, W.; Nazareth Rabello, M.; Pereira, C.A. & Ungaro, M.T. (1976). Cytogenetic study of DDT on human lymphocytes in vitro. *Mutation Research*, Vol.40, No.2, pp. 131-138, ISSN 0027-5107

Lindberg, H.K.; Wang, X.; Järventaus, H.; Falck, G.C.; Norppa, H. & Fenech, M. (2006). Origin of nuclear buds and micronuclei in normal and folate-deprived human lymphocytes. *Mutation Research*, Vol.617, No.1-2, pp. 33-45, ISSN 0027-5107

Martin, S.A.Jr.; Harlow, S.D.; Sowers, M.F.; Longnecker, M.P.; Garabrant, D.; Shore, D.L. & Sandler, D.P. (2002). DDT metabolite and androgens in African-American farmers. *Epidemiology*, Vol.13, No.4, pp. 454-458 ISSN 1044-3983

Mateuca, R.; Lombaert, N.; Aka, P.V.; Decordier, I. & Kirsch-Volders, M. (2006). Chromosomal changes: induction, detection methods and applicability in human biomonitoring. *Biochimie*, Vol.88, No.11, pp. 1515–1531, ISSN 0300-9084

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, pp. 663-671, ISSN 0027-8874.

McKenna, D.J.; Gallus, M.; McKeown, S.R.; Downes, C.S. & McKelvey- Martin, V.J. (2003). Modification of the alkaline Comet assay to allow simultaneous evaluation of mitomycin C-induced DNA cross-link damage and repair of specific DNA sequences in RT4 cells. *DNA Repair*, Vol. 2, No.8, pp. 879–890, ISSN 1568–7864

Meinert, R.; Schuz, J.; Kaatsch, P. & Michaelis, J. (2000). Leukemia and non-Hodgkin's lymphoma in childhood and exposure to pesticides: results of a register-based cite-control study in Germany. *American Journal of Epidemiology*, Vol.151, No.7, pp. 639–646, ISSN 0002-9262

Mellon, I.; Bohr, V.A.; Smith, C.A. & Hanawalt, P.C. (1986). Preferential DNA repair of an active gene in human cells, *Proceedings of the National Academy of Sciences of the United States of America,* Vol. 83, No.23, pp. 8878–8882, ISSN 0027–8424

Miller, GT. (2004). *Living in the environment*, Brooks/Cole Publishing, ISBN 978-0534997298, Pacific Grove, USA

Morgan, J.C.; Irving, H.; Okedi, L.M.; Steven, A. & Wondji, C.S. (2010). Pyrethroid resistance in an *Anopheles funestus* population from Uganda. *PLoS ONE,* Vol. 5, No. 7, e11872, ISSN 1932-6203

Morgan, D. & Roan C. (1974). The metabolism of DDT in man, In: *Essays in Toxicology,* W.Jr. Hayes, (Ed.), 39-97, Academic Press, ISBN 978-0121076054, New York, USA

Moriya, M. (1993). Single-stranded shuttle phagemid for mutagenesis studies in mammalian cells: 8-Oxoguanine in DNA induces targeted G.C–>T.A transversions in simian kidney cells. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.90, No. 3, pp. 1122–1126, ISSN 0027-8424

Moroy, Y.; Peebles, C.; Fritzler,M.J.; Steigerwald, J. & Tan,E.M. (1980). Autoantibody to centromere (kinetochore) in scleroderma sera. *Proceedings of the National Academy of Sciences of the United States of America,* Vol.77, No.3, pp. 1627–1631, ISSN 0027-8424

Natarajan, A.T.; Boei, J.J.; Darroudi, F.; Van Diemen, P.C.; Dulout, F.; Hande, M.P. & Ramalho, A.T. (1996). Current cytogenetic methods for detecting and effects of mutagens and carcinogens. *Environmental Health Perspectives*, Vol.104, No.3, pp. 445–448, ISSN 0091–6765

Natarajan, A.T. & Obe, G. (1982). Mutagenicity testing with cultured mammalian cells: cytogenetic assays, In: *Mutagenicity: New Horizons in Genetic Toxicology*, J.A. Heddle, (Ed.), 171-213, Academic Press, ISBN 978-0123361806, New York, USA

National Implementation Plan for the Stocholm Convention, Republic of Croatia. (2004). Available from http://www.cro-cpc.hr/projekti/pops/NIP_eng.pdf

Nevein, S.A. & Eman, M.S.Z. (2009). Detection of some organochlorine pesticides in raw milk in Giza Governorate. *Journal of Applied Sciences Research*, Vol.5, No.12, pp. 2520-2523, ISSN 1816-157X

Okonkwo, J.O.; Mutshatshi, T.N.; Botha, B. & Agyei, N. (2008). DDT, DDE and DDD in human milk from South Africa. *Bulletin of Environmental Contamination and Toxicology,* Vol. 81, No. 4, pp. 348-354, ISSN 0007-4861.

Olive, P.L. (1989). Cell proliferation as a requirement for development of the contact effect in Chinese hamster V79 spheroids. *Radiation Research*, Vol.117, No.1, pp. 79–92, ISSN 0033–7587

Olive, P.L.; Banáth, J.P. & Durand, R.E. (1990). Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay. *Radiation Research*, Vol.122, No.1, pp. 69-72, ISSN 0033–7587

Östling, O.; Johanson, K.J. (1984). Microelectrophoretic study of radiationinduced DNA damages in individual mammalian cells. *Biochemical and Biophysical Research Communications*, Vol.123, No.1, pp. 291–298, ISSN 0006–291X

Pérez-Maldonado, I.N.; Athanasiadou, M.; Yáñez, L.; González-Amaro, R.; Bergman, A. & Díaz-Barriga, F. (2006). DDE-induced apoptosis in children exposed to the DDT metabolite. *Science of the Total Environment*, Vol.370, No.2-3, pp. 343-351, ISSN 0048-9697

Pérez-Maldonado, I.N.; Díaz-Barriga, F.; de la Fuente, H.; González-Amaro, R.; Calderón, J. & Yáñez, L. (2004). DDT induces apoptosis in human mononuclear cells in vitro and is associated with increased apoptosis in exposed children. *Environmental Research*, Vol.94, No.1, pp. 38-46, ISSN 0013-9351

Pérez-Maldonado, I.N.; Herrera, C.; Batres, L.E.; González-Amaro, R.; Díaz-Barriga, F. & Yáñez, L. (2005). DDT-induced oxidative damage in human blood mononuclear cells. *Environmental Research*, Vol.98, No.2, pp. 177-184, ISSN 0013-9351

Peterson, J. & Robison, W. (1964). Metabolic products of p,p'-DDT in the rat. *Toxicology and Applied Pharmacology*, Vol.6, pp. 321-327, ISSN 0041-008X

Picer, M.; Picer, N.; Holoubek, I.; Klanova, J. & Hodak Kobasić, V. (2004). Chlorinate hydrocarbons in the atmosphere and surface soil in the areas of the city Zadar and Mt. Velebit Croatia. *Fresenius Environmental Bulletin*, Vol.13, No.8, pp. 712–718, ISSN 1018-4619

Pimentel, D. (2005). Environmental and economic costs of the application of pesticides primarily in the United States. *Environment, Development and Sustainability*, Vol.7, No.2, pp. 229-252, ISSN 1387-585X

Piperakis, S.M. (2009). Comet assay: a brief history. *Cell Biology and Toxicology*, Vol.25, No.1, pp. 1-3, ISSN 1573-6822

Popescu, N.C. (1999). Sister chromatid exchange formation in mammalian cells is modulated by deoxyribonucleotide pool imbalance. *Somatic Cell and Molecular Genetics*, Vol.25, No.2, pp. 101–108, ISSN 0740-7750

Rabello, M.N.; Dealmeida, W.F.; Pigati, P.; Ungaro, M.T.; Murata, T.; Perira, C.A. & Beçak, W. (1975). Cytogenetic study on individuals occupationally exposed to DDT. *Mutation Research*, Vol.28, No.3, pp. 449-454, ISSN 0027-5107

Ribas, G.; Surrales, J.; Carbonell, E.; Xamena, N.; Creus, A. & Marcos, R. (1996). Genotoxicity of the herbicides alachlor and maleic hydrazide in cultured human lymphocytes. *Mutagenesis*, Vol.11, No.3, pp. 221–227, ISSN 0267-8357

Rignell-Hydbom, A.; Lidfeldt, J.; Kiviranta, H.; Rantakokko, P.; Samsioe, G.; Agardh, C.D. & Rylander, L. (2009). Exposure to *p,p'*-DDE: a risk factor for type 2 diabetes. *PLoS ONE,* Vol.4, No.10, e7503, ISSN 1932-6203

Rupa, D.S.; Reddy, P.P. & Reddi, O.S. (1989). Frequencies of chromosomal aberrations in smokers exposed to pesticides in cotton fields. *Mutation Research*, Vol.222, No.1, pp. 37-41, ISSN 0027-5107

Rupa, D.S.; Reddy, P.P.; Sreemannarayana, K. & Reddi, O.S. (1991). Frequency of sister chromatid exchange in peripheral lymphocytes of male pesticide applicators. *Environmental and Molecular Mutagenesis*, Vol.18, No.2, pp. 136-138, ISSN 1098-2280

Rydberg, B.; Johanson K.J. (1978). Estimation of DNA strand breaks in single mammalian cells, In: DNA Repair Mechanisms, P.C. Hanawalt, E.C. Friedberg & C.F. Fox, (Eds.), 465-468, Academic Press, ISBN 9781555813192, New York, USA

Salama, S.A.; Serrana, M. & Au, W.W. (1999). Biomonitoring using accessible human cells for exposure and health risk assessment. *Mutation Research*, Vol.436, No.1, pp. 99-112, ISSN 1383-5742

Sannino, A.; Mambriani, P.; Bandini, M. & Bolzoni, L. (1996). Multiresidue method for determination of organochlorine insecticide residues in fatty processed foods by gel permeation chromatography. *Journal of AOAC International*, Vo.79, No.6, pp. 1434-1446, ISSN 1060-3271

Santos, S.J.; Singh, N.P. & Natarajan, A.T. (1997). Fluorescence in situ hybridization with comets. *Experimental Cell Research*, Vol.232, No.2, pp. 407–411, ISSN 0014-4827

Schaeferhenrich, A.; Sendt, W.; Scheele, J.; Kuechler, A.; Liehr, T.; Claussen, U.; Rapp, A.; Greulich, K.O. & Pool-Zobel, B.L. (2003). Putative colon cancer risk factors damage global DNA and TP53 in primary human colon cells isolated from surgical samples, *Food and Chemical Toxicology,* Vol.41, No.5, pp. 655–664, ISSN 0278–6915

Shaposhnikov, S.A.; Salenko, V.B.; Brunborg, G.; Nygren, J. & Collins, A.R. (2008) Single-cell gel electrophoresis (the comet assay): loops or fragments? *Electrophoresis*, Vol.29, No.14, pp. 3005-3012, ISSN 0173-0835

Shinmura, K. & Yokota, J. (2001). The OGG1 gene encodes a repair enzyme for oxidatively damaged DNA and is involved in human carcinogenesis. *Antioxidants & Redox Signaling,* Vol.3, No.4, pp. 597–609, ISSN 1523-0864

Singh N.P. (2000). A simple method for accurate estimation of apoptotic cells. *Experimental Cell Research*, Vol.256, No.1, pp. 328-337, ISSN 0014-4827

Singh, N.P.; McCoy, M.T.; Tice, R.R. & Schneider, E.L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research*, Vol.175, No.1, pp. 184-191, ISSN 0014-4827

Smith, C.C.; O'Donovan, M.R. & Martin, E.A. (2006). hOGG1 recognizes oxidative damage using the comet assay with greater specificity than FPG or ENDOIII. *Mutagenesis,* Vol.21, No.3, pp. 185–190, ISSN 0267-8357

Surrallés, J.; Falck, G. & Norppa, H. (1996). In vivo cytogenetic damage revealed by FISH analysis of micronuclei in uncultured human T lymphocytes. *Cytogenetics and Cell Genetics*, Vol.75, No.2-3, pp.151–154, ISSN 0301-0171

Sviežená, B.; Gálová, E.; Kusenda, B.; Slaninová, M.; Vlček, D.; Dušinská, M. (2004). The Comet assay and the troubles with its application in the green alga *Chlamydomonas reinhardtii*. *Czech Phycology,* Vol.4, No.1, pp. 163-174, ISSN 1802-5439

The European Standard EN 1258/1-4. (1996). *Fatty food: Determination of pesticides and polychlorinated biphenyls (PCBs), CEN/TC 275 N0360 Food analysis – Horizontal methods*

Thoday, J.M. (1951). The effect of ionizing radiation on the broad bean root. Part IX. Chromosome breakage and the lethality of ionizing radiations to the root meristem, *The British Journal of Radiology,* Vol.24, No.286, pp.276–572, ISSN 0007–1285

Thomas, P. & Fenech, M. (2011). Cytokinesis-block micronucleus cytome assay in lymphocytes. *Methods in Molecular Biology*, Vol.682, No.3, pp. 217-234, ISSN 1940-6029

Thomas, P.; Umegaki, K. & Fenech, M. (2003). Nucleoplasmic bridges are a sensitive measure of chromosome rearrangement in the cytokinesis-block micronucleus assay. *Mutagenesis*, Vol.18, No.2, pp. 187-194, ISSN 0267-8357

Thompson, E. J. & Perry, P. E. (1988). The identification of micronucleated chromosomes: a possible assay for aneuploidy. *Mutagenesis,* Vol.3, No.5, pp. 415-418, ISSN 0267-8357

Tice, R.R. (1995). Applications of the single cell gel assay to environmental biomonitoring for genotoxic pollutants, In: *Biomonitors and Biomarkers as Indicators of Environmental Change,* F.M. Butterworth, (Ed.), 314-327, Plenum Press, ISBN 9780306451904, New York, USA

Tice, R.R.; Agurell, E.; Anderson, D.; Burlinson, B.; Hartmann, A.; Kobayashi, H.; Miyamae, Y.; Rojas, E.; Ryu, J.C. & Sasaki, Y.F. (2000). Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environmental and Molecular Mutagenesis,* Vol.35, No.3, pp. 206-221, ISSN 0893–6692

Tirukalikundram, S.; Kumaravel, T.S. & Bristow, R.G. (2005). Detection of genetic instability at HER-2/neu and p53 loci in breast cancer cells using Comet- FISH. *Breast Cancer Research and Treatment,* Vol.91, No.1, pp. 89–93, ISSN 0167–6806

Tompić, T.; Šimunić-Mežnarić, V.; Posedi, M. & Božidar, B. (2011). Pesticides in food, *Hrvatski Časopis za Javno Zdravstvo*, Vol. 7, No. 25, ISSN 1845-3082

Torres-Sánchez, L. & López-Carrillo, L. (2007). Human health effects and p,p'-DDE and p,p'-DDT exposure: the case of Mexico. *Ciência & Saúde Coletiva*, Vol.12, No.1, pp. 51-60, ISSN 1413-8123

Turusov, V.; Rakitsky, V. & Tomatis, L. (2002). Dichlorodiphenyltrichloroethane (DDT): ubiquity, persistence, and risks. *Environmental Health Perspectives*, Vol.110, No.2, pp. 125-128, ISSN 0091-6765

United Nations Environment Programme/International Atomic Energy Agency. (1982). *Determination of DDTs, PCBs in selected marine organisms by gas-liquid chromatography*, Reference Methods for Marine Pollution Studies, No.17, UNEP

Uppala, P.T.; Roy, S.K.; Tousson, A.; Barnes, S.; Uppala, G.R. & Eastmond, D.A. (2005). Induction of cell proliferation, micronuclei and hyperdiploidy/polyploidy in the mammary cells of DDT- and DMBA-treated pubertal rats. *Environmental and Molecular Mutagenesis*, Vol.46, No.1, pp. 43-52, ISSN 1098-2280

Vaglenov, A.; Nosko, M.; Georgieva, R.; Carbonell, E.; Creus, A. & Marcos, R. (1999). Genotoxicity and radioresistance in electroplating workers exposed to chromium. *Mutation Research*, Vol.446, No.1, pp. 23–34, ISSN 1383-5718

Valko, M.; Izakovic, M.; Mazur, M.; Rhodes, C.I. & Telser, J. (2004). Role of oxygen radicals in DNA damage and cancer incidence. *Molecular and Cellular Biochemistry*, Vol.266, No.1-2, pp. 37-56, ISSN 0300-8177

van Wendel de Joode, B.; Wesseling, C.; Kromhout, H.; Monge, P.; García, M. & Mergler, D. (2001). Chronic nervous-system effects of long-term occupational exposure to DDT. *Lancet*, Vol.357, No.9261, pp. 1014-1016 ISSN 0140-6736

Wójtowicz, A.K.; Gregoraszczuk, E.L.; Ptak, A. & Falandysz, J. (2004). Effect of single and repeated in vitro exposure of ovarian follicles to o,p'-DDT and p,p'-DDT and their metabolites. *Polish Journa of Pharmacology*, Vol.56, No.4, pp. 465-472 ISSN 1230-6002

Wójtowicz, A.K.; Milewicz, T. & Gregoraszczuk, E.L. (2007). DDT and its metabolite DDE alter steroid hormone secretion in human term placental explants by regulation of aromatase activity. *Toxicology Letters*, Vol.173, No.1, pp. 24-30, ISSN 0378-4274

Wong, W.C.; Szeto, Y.T.; Collins, A.R. & Benzie, I.F.F. (2005). The comet assay: a biomonitoring tool for nutraceutical research. *Current Topics in Nutraceutical Research,* Vol. 3, No.1, pp. 1–14, ISSN 1540-7535

Woolcott, C.G.; Aronson, K.J.; Hanna, W.M.; SenGupta, S.K.; McCready, D.R.; Sterns, E.E. & Miller, A.B. (2001). Organochlorines and breast cancer risk by receptor status, tumor size, and grade (Canada). *Cancer Causes and Control*, Vol.12, No.5, pp. 395-404, ISSN 0957-5243

World Health Organization. (1990). *Public health impact of pesticides used in agriculture,* WHO, ISBN 9241561394, Geneva, Switzerland

World Health Organization. (2007). *Insecticide-treated mosquito nets: a WHO Position Statement*, WHO, Available from http://www.who.int/malaria/publications/atoz/itnspospaperfinal/en/index.html

World Health Organization. (2009a). *The WHO recommended classification of pesticides by hazard and guidlines to classification*, WHO, ISBN 9789241547963, Geneva Switzerland

World Health Organization. (2009b). *Global insecticide use for vector - borne disease control* (4th ed), WHO, ISBN 9789241598781, Lyon, France

Yáñez, L.; Borja-Aburto, V.H.; Rojas, E.; de la Fuente, H.; González-Amaro, R.; Gómez, H.; Jongitud, A.A. & Díaz-Barriga, F. (2004). DDT induces DNA damage in blood cells. Studies in vitro and in women chronically exposed to this insecticide. *Environmental Research*, Vol.94, No.1, pp. 18-24, ISSN 0013-9351

Part 2

Vector Management

7

Vector Control Using Insecticides

Alhaji Aliyu
Department of Community Medicine,
Ahmadu Bello University, Zaria
Nigeria

1. Introduction

At the end of the 19th century, it was discovered that certain species of insects, other arthropods and fresh water snails were responsible for the transmission of some diseases of pubic health importance. Since effective vaccines or drugs were not always available for the prevention or treatment of these diseases, control of transmission then had to rely mainly on control of vectors. The control programmes included among others, use of mosquito nets, drainage of gutters, filling of potholes and other water bodies used by insects for breeding. The 1940s sawed the discovery of DDT insecticide (dichlorodiphenyl tricloroethene) which was a major breakthrough in the control of vector-borne diseases. DDT also appeared to be effective and economical in the control of other biting flies (tsetse fly, simulium, and sand fly) and midges and of infestations with fleas, lice, bedbugs and triatomine bugs.

The initial large scale success achieved in the control programme was short-lived as the vectors developed resistance to the insecticides in use, thereby creating a need for new more expensive chemicals. Interest in alternatives to the use of insecticides such as environmental management (source reduction) and biological control, has been revived because of increasing resistance to the commonly used insecticides among important vector species e.g. (malaria) and also because of concerns about the effects of DDT and certain other insecticides on the environment. For many vector species, environmental sanitation[1] through source reduction and health education is the fundamental means of control; other methods should serve as a supplement, not as a substitute. Thus, in recent years, the practices of vector control have evolved, and environmental management and modification have come to the fore, both for disease control and for agricultural and other economic purposes[2], this is a complex and multi-disciplinary field[3]. Effective application of any control measure must be based on a fundamental understanding of the ecology, bionomics and behaviour of the target vector species and its relation to its host and the environment. Effective vector control also requires careful training and supervision of pest control operations and periodic evaluation of the impacts of the control measures.

In more recent years, less reliance has been placed on the use of a single method of chemical control; there is a shift towards more integrated vector control involving several types of environmental management supplemented by more than one method of chemical control and the use of drugs. More attention has been paid to community participation (a key component of primary health care (PHC) in eliminating breeding sites of vectors (including clearing of weeds/bushes near residential houses) and reducing vector densities.

Finally, there is also need to provide on continuous basis information on single, effective and acceptable methods for vector source reduction and personal protection to individuals and families in the community at a reasonable cost.
In a chapter of this nature only a few, but more important species of vectors could be discussed and even such a discussion could only be brief, emphasizing only the important features of the arthropod which serve to illustrate how the arthropod affects public health.

2. Malaria and its vectors

Human malaria is a number one public enemy and an illness caused by the bite of an infective female anopheles mosquito which transfers parasites called plasmodium from person to person. Four plasmodium parasites exists (P. falciparum, P. ovale, P. malariae and P. vivax) but only one (P. falciparum) is of vital importance in disease transmission in Nigeria. The important vectors in Nigeria are anopheles gambiae and A. funestus. These mosquitoes breed readily in ditches and collections of water in empty receptacles around the houses. The disease is endemic in Nigeria and over 90% of the population is at risk. Fifty percent of the population will have at least one attack per year, 300, 000 children and 11% of pregnant women die of malaria each year respectively and millions of dollars is lost each year in treatment of malaria.
The table below shows some of these vectors and diseases transmitted by them.

Type	Designation	Disease	Vectors	Mode of transmission
Protozoa	Plasmodium spp	Malaria	Mosquitoes	Bite (sg)
	Trypanosome spp	African sleeping sickness	Tsetse fly	Bite (sg)
Filarial nematodes	Wucheria Bancrofti Onchocerca	Filariasis	Mosquitoes	Bite
	Volvula	Onchocerciasis	Black flies	Bite
Viruses	YF	Yellow Fever	Aedes	Bite (sg)
Bacteria	Cholera/diarrhea	Cholera	Cockroaches	Mechanical carriers
	Typhoid fever	Typhoid fever		
	Bacillary dysentery	Dysentery		

Table 1. Pathogens and vectors of some human diseases (sg) indicates definite involvement of salivary gland.

The results of several surveys showed that the most prevalent species of malaria parasites is p. falciparum with a prevalent rate of between 80% and 100% of all positive blood films. P. vivax is conspicuously absent in West Africans who because of their Duffy negative erythrocyte membrane are immune to P. vivax[4, 5].
It is estimated that there are more than 400 species of anopheles in the world, but only 40 of these are important vectors (as transmitters or carriers) of malaria. Only the female anopheles mosquito transmits malaria to humans. Some anopheles mosquitoes can also carry other human diseases like Filariasis and some viruses. There are two other genera of mosquitoes which are important carriers of other mosquito-borne diseases: Aedes is a vector of viral diseases- Yellow fever and Dengue while culex mosquito is the vector of Filariasis

and Japanese encephalitis. For practical purposes and entomologically, it is useful to be able to differentiate between these three types of mosquitoes; so the main distinguishing features in each stage of the life cycle are shown below in the diagram

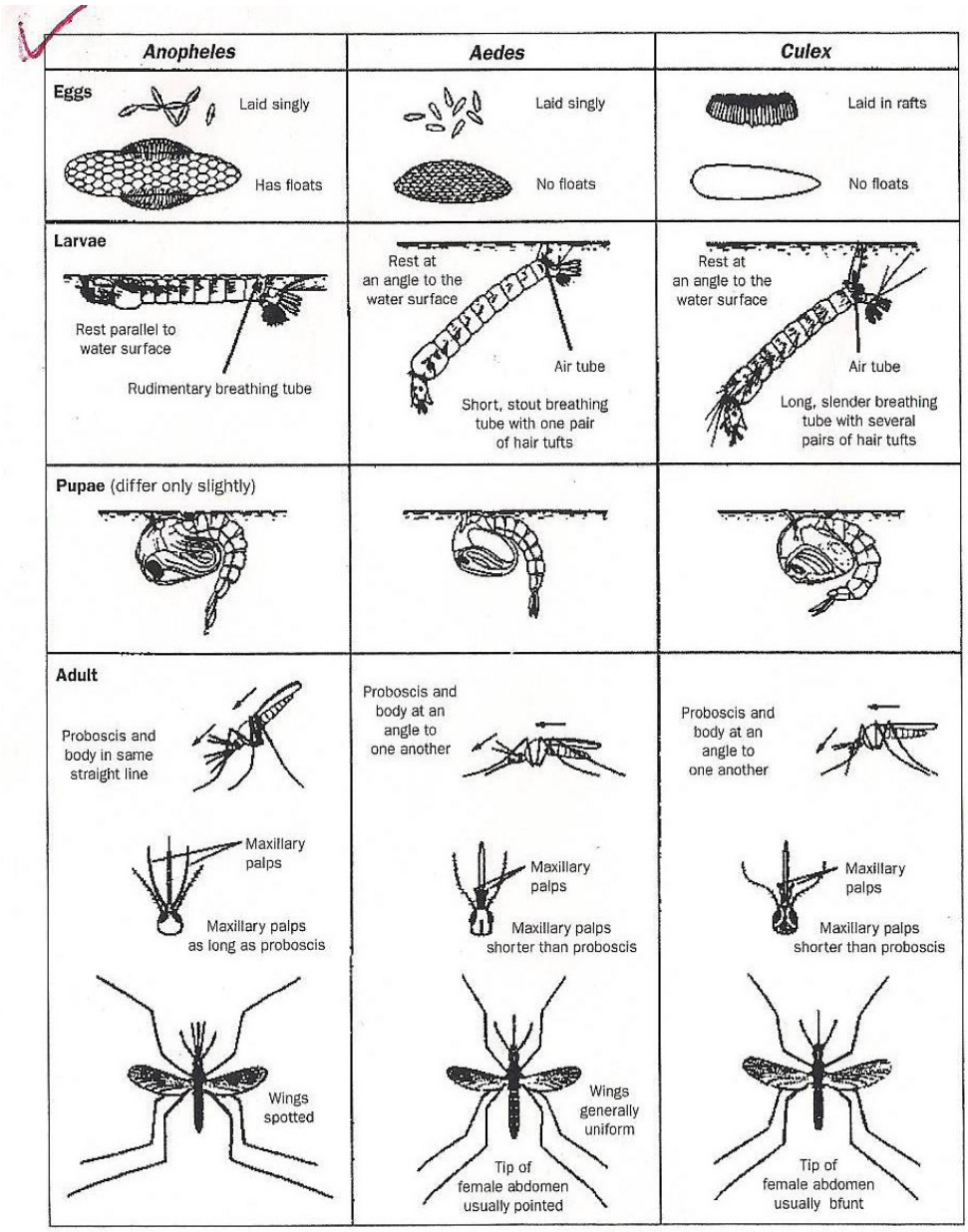


Fig. 1. Life cycles of different mosquito genera (online, 2011).

Basically, the mosquito has four stages in its life cycle: eggs → larva→ pupa → adult. It is useful to understand the life cycle and natural history of the anopheles mosquito not only for epidemiological reasons but most importantly for the fact that all the four stages are targets to control mosquito vector. There are several features of the behaviour of anopheles mosquitoes which are important in understanding malaria epidemiology and also for planning mosquito control.

Malaria is holo-endemic in most regions of Nigeria; ideal climatic conditions for the propagation and transmission of the infection are a temperature between 26^{o}C – 30^{o}C and relative humidity of over 60 percent.

3. Disease control

The general methods of malaria control can be grouped into three measures directed against the parasite in man, measures directed against the vector and measures designed to prevent mosquito-man contact, these are summarized in table 2

Principal goal	Interventions
Treatment	Outpatient treatment of uncomplicated malaria Inpatient treatment of severe and complicated malaria Home treatment
Prevention	
(a) Inhibit mosque ito breeding	i) Source reduction (drainage, filing in ditches) ii) Chemical larviciding iii) Management of agricultural, industrial and urban development to avoid breeding sites
(b)Kill adult mosquitoes	i) IRS (indoor residual spraying) ii) ITMs (insecticide treated materials – bed nets, curtains, etc)
(c) Prevent mosquito contact	i) ITMs ii) Repellants, sprays, coils, etc
(d) Reduce malaria infection and morbidity in humans	i) IPT (intermittent preventive treatment of pregnant women) ii) Chemoprophylaxis

Table 2. Interventions to control malaria[5].

The first involves the use of appropriate anti-malarial agents to treat clinical malaria and the use of chemoprophylaxis among the vulnerable groups. The problem facing most tropical countries is serious as majority of patients with clinical malaria are either untreated or treated inadequately by self-medication. Also, their governments cannot afford to buy sufficient anti-malarial drugs for their needs and most people cannot afford to purchase effective treatments[7]. The annual per capita expenditure on anti-malarial drugs in most of sub-Saharan Africa is still <US$10. An adult (60kg) course of Chloroquine costs US$0.08 but the new artemisinin based combinations (ACTs) cost more than five times as shown in table 3.

Drug	US$
Chloroquine	0.13
SP	0.14
Amodiaquine	0.20
Artemisinin-based combinations (ACTs)	1-3

Table 3. Average cost of a full course of adult outpatient treatment[8].

This is currently unaffordable to most patients surviving barely on less than a US$1 per day. The treatment of malaria has become quite challenging, and the emergence of resistant strains of the parasite. In Nigeria where at least 80%[9,10] of the people live in rural areas and are supperstitious illiterates, early recognition and the right treatment are not adhered to. This encourages drug resistance.

The Abuja declaration on Roll Back Malaria on 25 April 2000 and agreed to by African Heads of State sets an ambitious goals to reduce the burden of malaria (insecticide-treated nets, prompt access to treatment and prevention of malaria in pregnancy) by the year 2010[8]. Achieving high coverage in both IPT and use of ITNs among the vulnerable groups and the general population has remained elusive for many countries in sub-Saharan Africa[11]. A major barrier to net ownership is poverty as the price of a net represents a large proportion of the income of a poor household, this has been reported in various studies[12, 13, 14].

Environmental control (source reduction) offers the best practical and easy measure to control disease vector as it eliminates the breeding places. The filling of mosquito breeding sites with soil, ash or rubbish and is most suitable for reducing breeding in small depressions, water holes or pools, which does not require much filling material. On the other hand, drainage of water can be achieved by constructing open ditches; however, the drainage systems used in agriculture or for the transportation of sewage and rainwater in cities often promote breeding because of poor design and maintenance.

4. The use of insecticides

The first house-spraying campaigns after the Second World War, showed the capacity of this interventions to produce profound reductions in malaria transmission in a wide variety of circumstances. In Africa, the intervention was used in 1960s and 1970s but later abandoned except in some countries in southern and eastern Africa where residual insecticide spraying (IRS) remained the cornerstone of malaria control strategy. Since 2005, however, there has been renewed interest in large scale IRS programmes. To date, 25 out of the 42 malaria endemic countries in the WHO Africa region have included IRS in their control strategy for malaria control. Of these, 17 countries routinely implement IRS as a major malaria control intervention, six including Nigeria are piloting the IRS in a few districts while 2 are planning to pilot. At the end of 2006, the National Malaria Control Programme (NMCP) and its partners initiated a pilot IRS project in 3 local government areas/districts in 3 states (Lagos, Plateau, Borno states) using the WHOPES approved insecticides:

a. Lambdacyhalothrin CS 10% (0.030g/m^2)
b. Alphacypermethrin WP10% (0.030g/m^2)
c. Bifenthrin WP 10% (0.050g/m^2)

The evaluation of the local context of the 3 chemicals confirmed that residual effectiveness of the insecticides lasted for at least 4 months.

Residual spraying of houses involves the treating of interior walls and ceilings using a handheld compression spraying and is effective against mosquitoes that favour indoor resting before or after feeding. For a more detailed discourse of notable insecticide formulations, spray pumps, spraying techniques and maintenance of equipment, see Jan A. Rozendaal[15].
The figure 2, below summarizes and describes the IRS management principles;

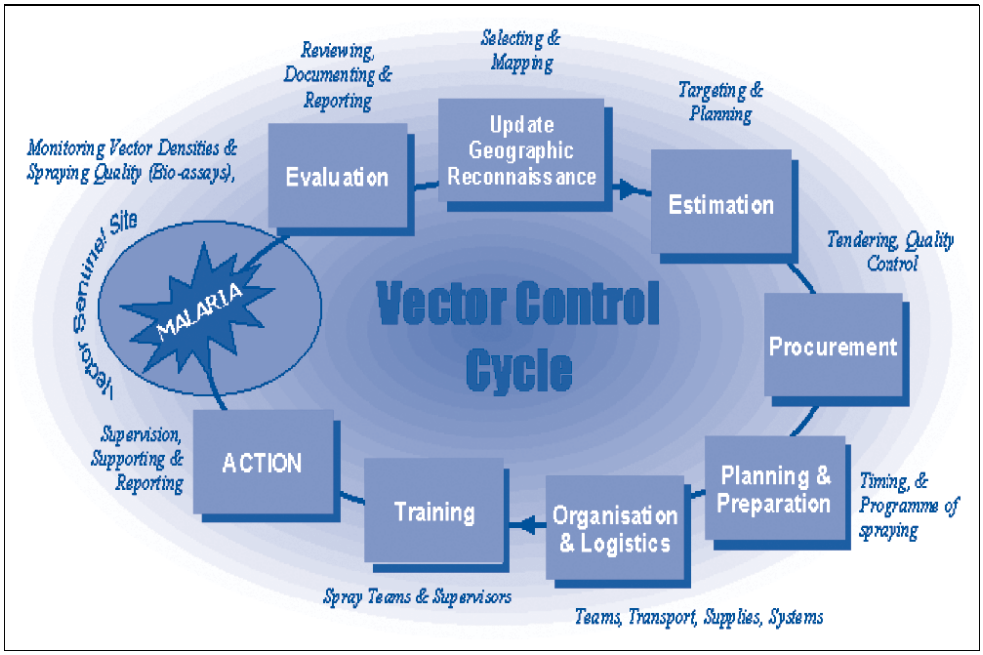


Fig. 2. IRS Management cycle.

Mosquito nets are an old technology and most people in sub-Saharan Africa are aware of the existence of nets. In some countries such as Nigeria and the Gambia nets have been in regular use for over hundred years. Similarly, in some parts of Africa, net ownership is a well established social norm and nets are widely available. For instance, recently, it was found that 70% of households (HHs) in Dar es Salaam (Tanzania) owned a mosquito net[16] and around 35% of HHs were found to own a net in urban Burkina Faso[17].
Insecticide treated mosquito nets have had significant impact in reducing morbidity and mortality among children under-five years old and pregnant women where ITNs have been appropriately and extensively used in malaria endemic areas. The potential epidemiological advantages and public health benefits of treating nets with insecticide for protection against malaria were recognized in the mid-1980s. Specifically, the efficacy of insecticide treated nets (ITNs) for the control of malaria in children under-5 years of age has recently been demonstrated by several large scale studies[18, 19, 20, 21] which find reductions in all cause – mortality, ranging from 16% to 63%. These insecticides which have been approved by WHOPES (World Health Organization Pesticides Evaluation Scheme) are safe. They have the following properties; provide personal protection from mosquito bites, effective against

other insects: bedbugs, flies and cockroaches, community and household mass effective which may be more important in some contexts[22] and mosquito nuisance effect. There have been reports also of dead insects on the nets and on the floor, less mosquito noise and that ITNs providing a better night's sleep than a net alone[16].

Most nets (and other materials) need to be treated and retreated with insecticides to increase their effectiveness. There are several insecticides that can be used which have proved to be safe. The insecticides recommended by the National Malaria and Vector Control Programme for treatment/retreatment of nets are those approved by WHOPES and registered by NAFDAC (National Foods and Drugs Administration Commission) are shown in table 4 below:

Generic name	Trade name	Dose per 1 net	Manufacturer
Alphacypermethrin 10%.SC[a]	Fendona	6ml	BASF
Deltamelthrin 1%.SC	K-Othrine, Ko-Tab	40ml	Aventis
Etofenprox 10%.EW[b]	Vectron	30ml	Mitsui
Permethrin 10%.EC[c]	Peripel, Imperator	75ml	Aventis
Cyfluthrin 5% EW	Solfac EW	15ml	Bayer
Lambdacyhalothrin 2.5% CS[d]	Icon	10ml	Syngenta

Key:

SC[a] = Suspension concentrate
EW[b] = Emulsion oil in water
EC[c] = Emulsifiable concentrate
CS[d] = Capsule suspension

Table 4.

For a detailed description of preparation of insecticide mixtures and treatment methods, the reader should consult guidelines on the use of insecticide-treated mosquito nets[23].

A single impregnation of a cotton or nylon mosquito net will provide protection for 1 year[24,25]. Nylon tends to retain permethrin and deltamethrin better than cotton. The impregnated nets can be washed and can tolerate small tears/holes without markedly reducing the protective effect. Recently, long lasting nets (LLNs) have been developed and have the advantage of retaining insecticidal activity for years (so the nets will not lose its potency with repeated washings). Despite various government policies, cost of the nets remains a significant barrier and a long obstacle to the Roll Back Malaria goal of universal coverage – defined as one long lasting insecticide-treated nets for every two people in the household with 80% usage. ITN development is a public good. The development of insecticide resistance in the 1950s and recently by vectors has been a cause for concern. There have been reports of resistance to DDT in a wide range of sub-Saharan African countries[26], but this has not reached an operationally significant level. With the exception of the Gezira region of Sudan[27], widespread loss of vector susceptibility is not yet a big problem in Nigeria. A worrying new development is the emergence of knockdown resistance to pyrethroid insecticides in natural populations of anopheles mosquitoes in Cote d'Ivoire and Burkina Faso[28, 29, 30] where insecticides are widely used in cotton production.

Currently, pyrethroids are the only insecticides used for net treatment and are also increasingly used for spraying, so there is threat that if widespread resistance develops, the interventions will gradually become less cost-effective over time.
Finally, there is need to describe importantly the WHO integrated vector management (IVM) concept. Vector control is well suited for integrated approaches because vectors are responsible for multiple diseases and since interventions are effective against several vectors (use of insecticides) the concept of IVM was developed as a result of lessons learnt from integrated pest management which was used in Agriculture.
Integrated vector management (IVM) is a major component of the global campaign against malaria. The revised strategic plan for RBM recommended that from 2006 – 2010, 80% of the population at risk need to be protected using effective vector control measures. IVM creates synergies between various vector-borne disease control programmes. Utilization of single method could be optimized to control more than one vector-borne disease, eg ITNs can control malaria, lymphatic filariasis and to some extend leishmaniasis. IVM operates in the context of inter-sectoral collaboration. The application of IVM principles to vector control will contribute to the judicious use of insecticides and extend their useful life.

5. References

[1] World Health Organization. Chemical methods for the control of arthropod vectors and pests of Public Health importance. WHO, Geneva; 1984.
[2] Najera J A. Malaria control: achievements, problems and strategies. Parasitologica 2001, 43:1-89
[3] Molineaux I, Grammiccia G: The Garki project; WHO 1980
[4] Garnham P.c.C. (1971).Progress in parasitology. The Atlone Press, London
[5] Kara Hanson, Catherine Goodman, Jolines, Sylvia Meek, David Bradley and Anne Mills. Global Forum for health Research: The Economics of Malaria Control Interventions, 2004.
[6] Stages of life cycle of vector parasites, available at www.who.int/docstore/water_control_htlm (cited 11/05/2011)
[7] Miller, L.H., Mason, S J, Clyde, DF and McGinnis M H. Resistance factors to plasmodium vivax: Duffy genotype Fyfy. New England Journal of Medicine: 1976; 255:302-304
[8] Denis MB, Tsuyuoka R, Poravuth Y et al. Surveillance of the efficacy artersunate and mefloquine combination for the treatment of uncomplicated malaria in Cambodia. Tropical Medicine International health 2006; 11:1160-1366
[9] WHO. The Africa Malaria Report 2003, WHO/CDS/MAL/2003/1903:31-37
[10] Salako A A. Environmental health education in schools and communities in Nigeria: The essence. The Nigerian Clinical Review Journal (2008): 14; 12:17-25
[11] The Challenges of Malaria Infection in Nigeria: The Nigerian clinical Review. March/April 2011; 89:3-6.
[12] Anna Maria van Eijk, Jenny Hill, Victor A. Alegana et al. Coverage of Malaria protection in pregnant women in sub-Saharan Africa: A synthesis and analysis of National survey data. Medi-Link Journal. April 2011: 12:4:20-42

[13] Aliyu A A, Alti-Mu'azu M, Insecticide-treated net usage and malaria episodes among boarding students in Zaria, Northern Nigeria. Annals of African Medicine, 2009; 8:2:85-89.

[14] Aniefiok M. Acceptance and use of insecticide treated bed nets (ITN in the Roll Back Malaria Programme in Kuje Area Council, Abuja. Journal of Environmental health 2005; 2:26-33

[15] Insecticide-treated nets. New findings from Kenya, published by Centers for Disease Control and Prevention (CDC), Atlanta, USA, Confirm effectiveness in areas of intense transmission. Africa Health (Mera) July 2003; Issue 6

[16] Jan A. Rozendaal: Vector control (Methods used by individuals and communities) 1997.

[17] Jones C. Bed nets and malaria. Postgraduate Doctor. May 2002; vol. 24 No. 2:23-25.

[18] Guiguemde T R et al. Household expenditure on Malaria prevention and treatment of families in the town of Bobo-Diolasso, Burkina Faso. Trans. of the Royal Society of Tropical Medicine and Hygiene 1994: 88: (3)285- 287

[19] Alonso P L, Lindsay SW, Schellenberg J. et al. A malaria control net trial using insecticide-treated bed nets and targeted chemoprophylaxis in a rural area of the Gambia, West Africa. The impact of the interventions on mortality and morbidity from malaria. Transactions of the Royal Society of Tropical Medicine and Hygiene, 1993; 87(S):37-44.

[20] D'Alessandro U, Olaleye BO, McGuire W et al. Mortality and Morbidity from malaria in Gambian children after introduction of an impregnated bed net programme. Lancet 1995:345 (8948).479-83

[21] Binka F N, Kubaje A, Adjuik M et al. Impact of permethrin impregnated bed nets on child mortality in Kassena-Nankana district, Ghana: A randomized controlled trial. Tropical Medicine and International health 1996; 1(2):147-54.

[22] Nevill CG, Same ES, Mung'ala VO et al. Insecticide-treated bed nets reduce mortality and severe morbidity from malaria among children on the Kenyan Coast. Tropical Medicine and International Health 1996: 1(2) 139-46

[23] Nicholas J. White. Malaria Gordon C. Cook and Alimuddin I Zumla (Eds). Manson's Tropical Diseases! 22nd edition, China, Saunders Elsevier: 2009; 1201-1300

[24] WHO Guidelines on the use of insecticide-treated nets for the prevention and control of malaria in Africa.

[25] Lindsay SW, Gibson MF. Bed nets revisited – old idea, new angle. Parasitology Today 1988:4:270-272

[26] Alonso PI, Lindsay SW, Armstrong IRM, et al. The effects of insecticide-treated bed nets on mortality of Gambian children. Lancet 1991;337:1499-1502

[27] WHO Vector resistance to pesticides, Fifteenth Report of the WHO Expert Committee of Vector Biology and Control, Geneva; 1992

[28] El Gaddal A A, Haridi A A M, Hassan F T, Hussein H. Malaria Control in Gezira-Managil Irrigated Scheme of the Sudan. Journal of Tropical Medicine and Hygiene 1985; 88:153-157

[29] Martinez Torres D, Chandre F, Willamson M S et al. Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector anopheles Gambiae SS. Insect Molecular Biology 1998; 7(2):174-184

[30] Elissa N, Morchet J, Riviere F, Mevnier J-Y, Yaok. Resistance of Anopheles gambiae SS to pyrethroids in Cote d'Ivoire. Annales de la Societe Belge de Medecine Tropicale 1993; 73:291-294.

8

Susceptibility Status of *Aedes aegypti* to Insecticides in Colombia

Ronald Maestre Serrano
Doctoral Student in Tropical Medicine
Cartagena University
Colombia

1. Introduction

Dengue is a disease of public health interest for Colombia due to its impact on morbidity and mortality. This disease mantains an endemic behavior in the departments of the country located below 2200 meters above sea level. Between the years 1978 to 2010 it was recorded approximately a number of 1,011,852 cases of dengue in Colombia. Vector populations of *Aedes aegypti* in the country have been continuously pressed for over five decades with insecticides, being the most widely used tool for interrupting transmission of dengue virus during outbreaks and epidemics in the absence of an available vaccine (Figure 1). The selection pressure excercised with these chemicals has led to the emergence of resistant populations of the vector to organochlorine, organophosphate, pyrethroid and carbamate-like molecules.

Fig. 1. Application of insecticides in the home by fogging durin outbreaks or epidemics.

The records of vector resistance in the country have increased considerably since 2007 when in Colombia was created the national network for the surveillance of susceptibility to insecticides used in public health to *A. aegypti* and major vector of malaria. With this information a base line has been defined for some Colombian departments in the region and basic knowledge has been generated for decision making from the operating standpoint of vector control programs. However, more research on issues related to the enzymatic mechanisms and molecular causes of resistance in vector populations is required as most results to date have been from diagnostic doses through standardized techniques by the Centers for Disease Control and Prevention (CDC) and World Health Organization (WHO). In this chapter these results are socialized and we discuss some ideas related to the topic.

2. Study area

The continental territory of the Republic of Colombia is located in the northwest corner of South America on the equator. Colombia is located in the north to 12°26'46'' north latitude at the place called Punta Gallinas in the peninsula of Guajira. To the south, is located at 4° 12' 30'' de latitude south, the East is located at 60°50'54'' west longitude from Greenwich, by the West is located at 79°02' 33'' west longitude from Greenwich.

Colombia territory also includes the archipelago of San Andres and Providencia in the Caribbean sea between 12° and 16°30´north latitude and 78° and 82° west longitude of Greenwich. Colombia has a land area of 1.141.748 km² ranking fourth among the countries of South America.

Colombia is a country with a wide variety of climates, from the coldest to 0°C to temperatures exceeding 28°C especially in the Caribbean regions, Pacific, Amazon and the Orinoco, accounting for nearly 80% of Colombia territory. Colombia is divided administratively into 32 states, these in turn are divided into 1102 municipalities.

3. The situation of dengue in Colombia

In Colombia, dengue is a disease of public health interest for its impact on morbidity and mortality. Its endemic behavior is due to endemic multiple factors including the re-emergence and intense viral transmission with a rising trend, the behavior of epidemic cycles getting shorter, the increased frequency of outbreaks and serious of the disease, the simultaneous circulation of the four serotypes, infestation by *A. aegypti* in over 90% of the national territory located below the 2,200 meters, and other social factors such as uncontrolled increase and lack of basic sanitation in urban centers (Escobar, 2009). The epidemiological pattern of disease in the last decades has been upward, characterized by exponential increase in endemic areas during the different decades. Cyclical behavior has been characterized by epidemic peaks every three or four years, dealing with the reentry of new serotypes to the country.

In Colombia from 1978 to 2010, has officially been recorded a total of 1.011.852 cases of dengue. For this time period there is record history of the following epidemics in the country:

1977: First recorded epidemic of dengue in Colombia.

2002: Epidemic in 76,996 cases, of which 5269 corresponded to Dengue hemorrhagic fever.

2007: Epidemic in 43,227 cases, of which 4665 corresponded to Dengue hemorrhagic fever.

2010: It is recorded in the country the worst epidemic of this disease with 157,152 confirmed cases and 217 deaths.

The departments that have historically had more transmission of dengue in the country are: Atlántico, Santander, Norte de Santander, Valle del Cauca, Antioquia, Tolima, Huila, Cundinamarca, and Casanare.

4. Resistance to insecticides

4.1 Definition

Resistance is defined as the development of the ability to tolerate doses of toxics, which can be lethal to most individuals in a normal population of the same species and is the result of positive selection pressure exerted by the insecticidal over genes initially in low frequency (WHO, 1957).

4.2 Mechanisms of resistance

The two main resistance mechanisms are alterations in the target site and metabolic resistance, also called increase in the rate of detoxification of insecticides. However, there are other less frequent as there are the resistance behavior and decreased penetration. (Miller, 1988; Bisset, 2002; Fonseca and Quiñones, 2005)

4.2.1 Altered target site

This is generated when no silent mutations occur in structural genes that generate a disturbance of amino acids responsible of anchor of the insecticide at a specific site.

4.2.1.1 GABA Receptor

In bugs this is an hetero multi numeric channel acting as a site of action for cyclodiene and avermectins. The resistance is given by *Rdl* gene (Resistance to dieldrin), which encodes for RDL, a GABA receptor subunit. This type of resistance was first identified in *Drosophila* first identified this type of resistance (Fonseca and Quiñones, 2005).

4.2.1.2 Voltage-gated sodium channel

They are the target of action of pyrethroids and DDT. The sodium para-channel protein is a complex of over 2000 amino acids, composed of 4 homologous domains separated by hydrophilic links, each domain contains six segments. The first mutation in the sodium channel that conferred resistance was detected in *Musca domestica*. subsequently mutations in other insects have been identified including Culicidae resistant to pyrethroids (Martínez et al, 1998; Brengues et al, 2003; Anstead, 2005; Saavedra et al, 2007; Brooke, 2008; Chang et al 2009; Yanola et al, 2010).

4.2.1.3 Insensitive acethylcholinesterase

Insecticide resistance, attributed to Ache insensitivity is found in a number of *Anopheles* and *Culex* species. In general, this mechanism produces a broad spectrum resistance to most organophosphates and carbamates, although more pronounced to carbamatos. (Bisset, 2002).

4.2.2 Metabolic resistance

It is conferred by an increase in detoxification insecticide or an inability to metabolize the toxic compound. The most important form of metabolic resistance is given by detoxificating enzymes of the type glutathione S-transferase, mixed function oxidases and esterases (Bisset 2002).

4.2.2.1 Carboxylesterases

They Catalyze the hydrolysis of carboxylic esters and changes in their expression levels, this is the resistance mechanism that occurs most often in insects. In mosquitoes, high levels of these enzymes have been associated with resistance to organophosphate and pyrethroid insecticides (Cui et al, 2007; Rodriguez et al, 2007). Esterases is a family of six proteins

Esterases are a family of six proteins grouped in α and β hydrolases superfamily. In the Diptera these enzymes are encoded by a gene cluster on the same chromosome, each gene may suffer modification that confers resistance to the insecticide (Fonseca and Quiñones, 2005; Santacoloma, 2008).

4.2.2.2 P450 Enzymes or mixed function oxidases

P450 enzymes are encoded by cytochrome P450 genes. P450 enzymes also have other names such as: cytochrome P450 monooxygenases, mixed function oxidases, monooxygenases with polisustrato, microsomal oxidases and heme-thiolate proteins. In insects these enzymes are involved in growth and development through the processing of fatty acids, hormones and pheromones; in the metabolism of secondary plants products and synthetic chemicals such as insecticides. The cytochrome P-450 is implicated as the major factor in many cases of metabolic resistance to carbamates and detoxify organophosphates, pyrethroids and DDT as well. It expresses in tissues of the gut, fat, reproductive tract and Malpighian tubes (Feyereisen, 1999; Poupardin et al, 2008).

4.2.2.3 Glutatione s-transferase

The glutathione s-transferases (GSTs, EC 2.5.1.18) are phase II enzymes involved in xenobiotics detoxification in many organisms. These enzymes metabolize a wide range of toxic hydrophobic compounds such as drugs, insecticides and endogenous toxic substrates, catalyzing the conjugation of glutathione to the hydrophilic center of toxic substances, allowing the increase in compounds solubility. The GSTs are divided into three main groups: the cytosolic, microsomal and mitochondrial. The mitochondrial GSTs have not been found in insects species including mosquitoes. The cytosolic GSTs in insects are grouped into six different kinds: Delta, Epsilon, Omega, Sigma, Theta and Zeta. Most GSTs involved in the metabolism of xenobiotics in insects belong to the class Delta Epsilon. GSTs in insects have been implicated in the insecticide resistance through its direct metabolism, sequestration or protection against secondary toxic effects such as increased lipid peroxidation induced by exposure to insecticides. Glutathione S- transferase mediated detoxification has been reported for insecticides of the organophosphate type and DDT. The main role of GSTs in the resistance to organophosphates is the detoxification of the insecticide by a conjugation reaction. The specific GSTs also catalyze the metabolism of DDT to a non-toxic substance: 1,1-dichloro-2 ,2-bis-(p-chlorophenyl) ethane (DDE), through the process of dehydrochlorination. Epsilon class of GST has been involved in resistance to DDT in *Anopheles gambiae* and *A. aegypti* (Lumjuan et al, 2007).

4.3 Type of resistance

4.3.1 Cross resistance

It occurs when a single gene confers resistance to a number of chemicals in the same group, as it is the case of phosphotriesterases that provides resistance to several organophosphates or *kdr* gene that confers resistance DDT and the pyrethroids (WHO, 1957).

4.3.2 Multiple resistance

It occurs when two or more resistance mechanisms are operating in the same insect. The multiple resistance term does not necessarily involve the term cross-resistance, because a bug may be resistant to 2 or more insecticides and each resistance can be attributed to different mechanisms (Bisset, 2002).

4.4 Techniques used to detect resistance insecticides in mosquitoes

4.4.1 Biological test of the World Health Organization (WHO)

Technique standardized by the World Health Organization (WHO). In adult mosquitoes the technique consists of exposing individuals of the populations we intent to evaluate to papers impregnated with a single and specific dose of an insecticide during a determined time (1 hour with the exception of the organophosphate fenitrothion, to which will be exposed for 2 hours). The mosquitoes are then transfered to a paper that hasn't been impregnated, where they rest under controlled conditions of temperature, relative humidity and an energy source consisting of a sugar solution (Figure 2A). The mortality reading is done after 24 hours of exposure to the insecticide (WHO 1981a).

The susceptibility of larvae is measured by exposing individuals to a diagnostic concentration or several temephos concentrations to determine the lethal concentration (LC_{50} or CL_{95}) to calculate resistance factor using water as solvent (Figure 2B). Individuals remain exposed for 24 hours. (WHO 1981b) (Santacoloma, 2008).

Fig. 2A. Biological test of the World Health Organization (WHO) (mosquitoes).

4.4.2 Test for biological control center and prevention (CDC)

The impregnated bottles technique was standardized by the Center for Diseases Control and Prevention (CDC) (Brogdon and McAllister, 1998). The objective of this technique is to measure the time it takes dose of an insecticide to reach the target site of action in the

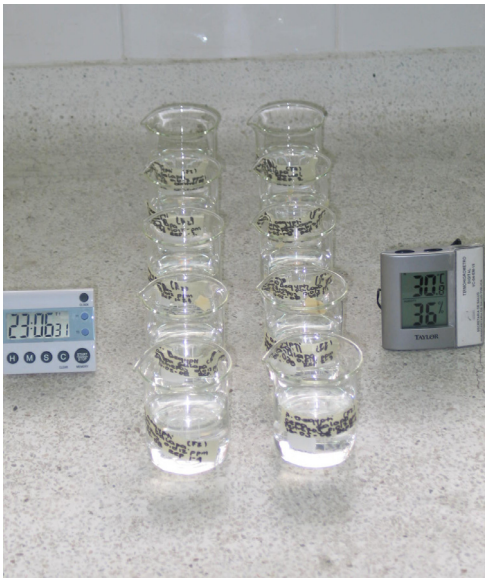


Fig. 2B. Biological test of the World Health Organization (WHO) (Larvae).

mosquito. Involves subjecting a sample of mosquitoes between 15 and 25 individuals of the population to be evaluated on a glass surface pre-impregnated with the dose of insecticide. (Santacoloma, 2008) (Figure 3).

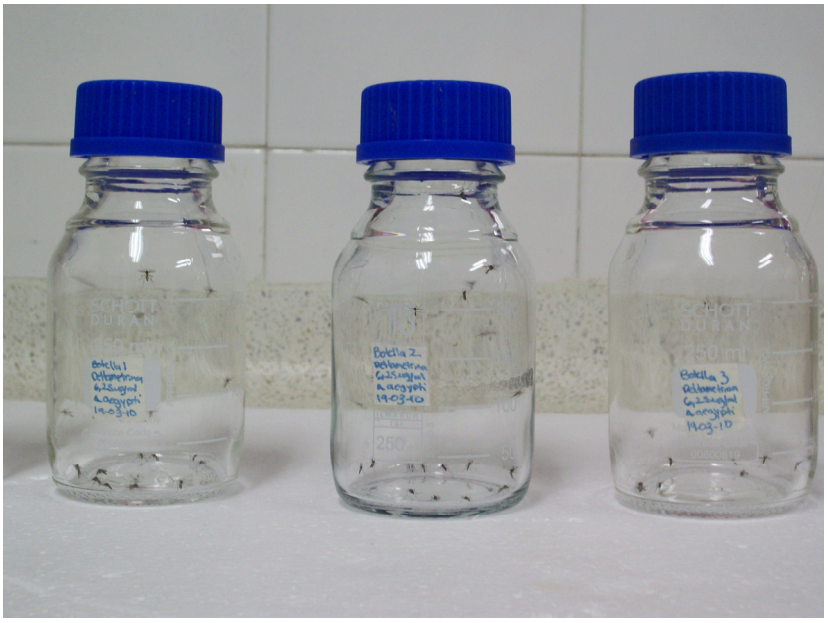


Fig. 3. Test for biological control center and prevention (CDC).

4.4.3 Synergists

These are chemicals that specifically inhibit insecticide-metabolizing enzymes, enhancing their action. Among the synergists more used to detect resistance mechanisms in Insects are the S, S, S - tributilfosforotioato (DJF) and esterases inhibitor and of the enzyme glutathione transferase (GST), the triphenyl phosphate (TFF) specific esterase inhibitor, piperonyl butoxide (PB), an inhibitor of monooxygenases, and ethacrynic acid (EA), a specific inhibitor of the enzyme glutathione transferase (GST) (Rodríguez, 2008).

4.4.4 Biochemical test

The biochemical assays are used to define metabolic mechanisms that may be responsible for the physiological resistance in an insect population (WHO, 1992). The metabolic mechanisms include tests to determine the target enzyme decreased sensitivity or the increased enzyme activity. For the first mechanism in particular, measures the change in acetylcholinesterase associated with resistance to carbamates and organophosphates. For the second, evaluates the increased activity of esterases, mixed function oxidases and glutathione s-transferase for kidnapping or increased detoxification of insecticides (Santacoloma, 2008).

4.4.5 Molecular tests

These tests consist in the amplification of specific gene sequences through polymerase chain reaction technique (PCR) to detect mutations.

4.5 Current state of susceptibility to insecticides *Aedes aegypti* in Colombia

Since the late forties, when first reported resistance to DDT in *Aedes tritaeniorhynchus* (Weidemann) and *Aedes solicitans* (Walker) resistance has been recorded in over a hundred species for one or more insecticides of public health use worldwide (Brown, 1986; Fonseca & Quiñones, 2005). For *A. aegypti* in America resistance has been reported to organochlorines, organophosphates, pyrethroids and carbamates in Argentina, Brazil, Mexico, El Salvador, Peru, Panamá, Venezuela, Cuba, Puerto Rico, among other Caribbean countries, whose resistance mechanism in some of these stocks has been associated with altered levels of alpha esters, beta esterases, mixed function oxidases, glutathione s- transferase, as well as mutations in the voltage-gated sodium channel. (Rawlins, 1998; Bisset et al, 2001; Brengues et al, 2003; Macoris et al, 2003; Aparecida et al, 2004; Rodríguez et al, 2004; Chavez et al; 2005, Flores et al, 2005; Pereira da-Cunha et al, 2005, Alvarez et al, 2006, Pereira-Lima et al, 2006; Beserra et al, 2007; Saavedra et al, 2007; Bisset et al, 2009; Martins et al, 2009; Albrieu-Llinas et al, 2010; Polson et al, 2010).

Colombia has applied insecticides for control of vector insects for over five decades. The DDT was the first applied to control malaria and during the campaign for the eradication of *A. aegypti* conducted in the early 1950. This insecticide was banned in the late 60's, due to the findings of resistance worldwide (Brown, 1986). Since 1970, organophosphates including temephos were applied and from the early 90's the use of pyrethroids was started. From that time on, the country has been rotating the application of molecules for mosquito control such as: deltamethrin, lambda-cyhalothrin, malathion, fenitrothion and in the last three years cyfluthrin and pirimiphos-methyl. However, the resistance to these insecticides has been documented gradually, making it difficult to take control actions within programs of Vector Borne Diseases in different regions of the country.

In Colombia until the 1990's there were few works assessing the state of susceptibility in Culicidae populations of interest in public health. Between 1959 and 1987 the first cases of DDT resistance were registered in populations of *Anopheles albimanus* (Wiedemann) in the municipalities of El Carmen (Bolivar); Codazzi, Robles and Valledupar (Cesar); Acandí (Choco) and *An. darlingi* (Root) in some locations of Quibdó municipality (Chocó) (Quiñones et al, 1987). Later Suarez et al, (1996) recorded in the 90's decade the first case of resistance to temephos in the *A. aegypti* species in Cali, Valle del Cauca, and Bisset et al, (1998) evaluated the susceptibility in a strain of *Culex quinquefasciatus* (Say) from Medellin, Antioquia, encountering resistance to organophosphates malathion, primifos-methyl, chlorpyrifos, temephos, fenthion and pyrethroids deltamethrin and permethrin.

In the absence of enough studies in Colombia on susceptibility status of populations of *A. aegypti* to several insecticides of use in public health and in compliance with public policies enshrined in the American continent resolutions CD39.R11 1996, CD43R4 2001 of the Pan American Health Organization, during the years of 2005 and 2007 a national project was conducted, this was funded by Colciencias (Colombian Science and Research Organization) and implemented by the Learning and Control of Tropical Diseases Program (PECET) of the University of Antioquia, the International Centre for Training and Medical Research (CIDEIM), the National University in Colombia, the National Institute of Health (NIH) and 12 departments of health seeking to generate baseline susceptibility of vector populations in Colombia. This multicentered project gave rise to the national surveillance network susceptibility to insecticides for *A. aegypti* and main vectors of malaria led by the National Institute of Health (INS). Since then the record of resistance to *A. aegypti* in Colombia expanded through biological tests by the CDC and WHO as well as the determination of impairment of enzymes involved in resistance.

With these results it has been observed for Colombia widespread resistance to DDT (Figure 4A-4B) and variability in susceptibility to the following insecticides: temephos, lambda-cyhalothrin, deltamethrin, permethrin, cyfluthrin, etofenprox, malathion, fenitrothion, pirimiphos methyl, bendiocarb and propoxur in different regions, with deterioration in some populations in the levels of nonspecific esterases, mixed function oxidases and in smaller proportion to glutathione s-transferesas (Figure 5A, 5B, 5C, 5D) (Rojas et al, 2003; Cadavid et al, 2008; Fonseca et al, 2006; Fonseca et al, 2007; Orjuela et al; 2007, Salazar et al, 2007; Santacoloma et al, 2008; Fonseca et al, 2009; Maestre et al, 2009; Maestre et al, 2010; Ardila and Brochero, 2010, Gomez et al, 2010; Maestre et al, 2010, Fonseca et al, 2011).

For temephos resistance has been observed in Cundinamarca, Guaviare, Meta, Santander, Cauca, Valle del Cauca, Nariño, Huila, Caldas, Sucre, Atlantico, La Guajira. (Anaya et al, 2007; Santacoloma, 2008; Maestre et al, 2009; Ocampo et al, 2011) (Figure 6).

Pyrethroids that despite being used in Colombia more recently compared to organophosphates, have shown higher levels of resistance despite the increased use time of organophosphate (Figures 7A, 7B, 7C, 7D, 7E), (8A, 8B, 8C). Among the pyrethroids lambda is the insecticide wich displays higher frequency of resistance in vector populations in the country. However, there are pyrethroids such as permethrin and etofenprox that despite having no use in public health have resistance generated in populations of *A. aegypti* from Casanare, Antioquia, Chocó and Putumayo (Ardila and Brochero, 2010; Fonseca et al, 2011).

For the carbamate propoxur discordance in susceptibility results was observed between the WHO technique in which resistance and CDC susceptibility is recorded. Further studies are required to determine the state of populations' susceptibility to the insecticide (Fig. 9 AB). Furthermore, few studies in the country have evaluated the susceptibility of the vector to the

insecticide Bendiocarb to which there has been resistance registered in the populations in the department of Cauca, Valle del Cauca, Huila and Nariño (Figure 10 AB) (Ocampo et al, 2011).
Currently it has not been registered for the country mutations in voltage-gated sodium channel gene. It is therefore recommended studies to perform studies to explain resistance observed to most of the pyrethroids evaluated in different country populations and may be related to a crossed resistance to DDT. It is also recommended to perform studies to determine cross resistance to other molecules such as organophosphate and carbamate, as well as multi resistance studies.
For Colombia it is recommended to maintain a system of permanent time and space surveillance that allows health authorities to use insecticides with technical criteria to maintain effective control interventions in vector populations.

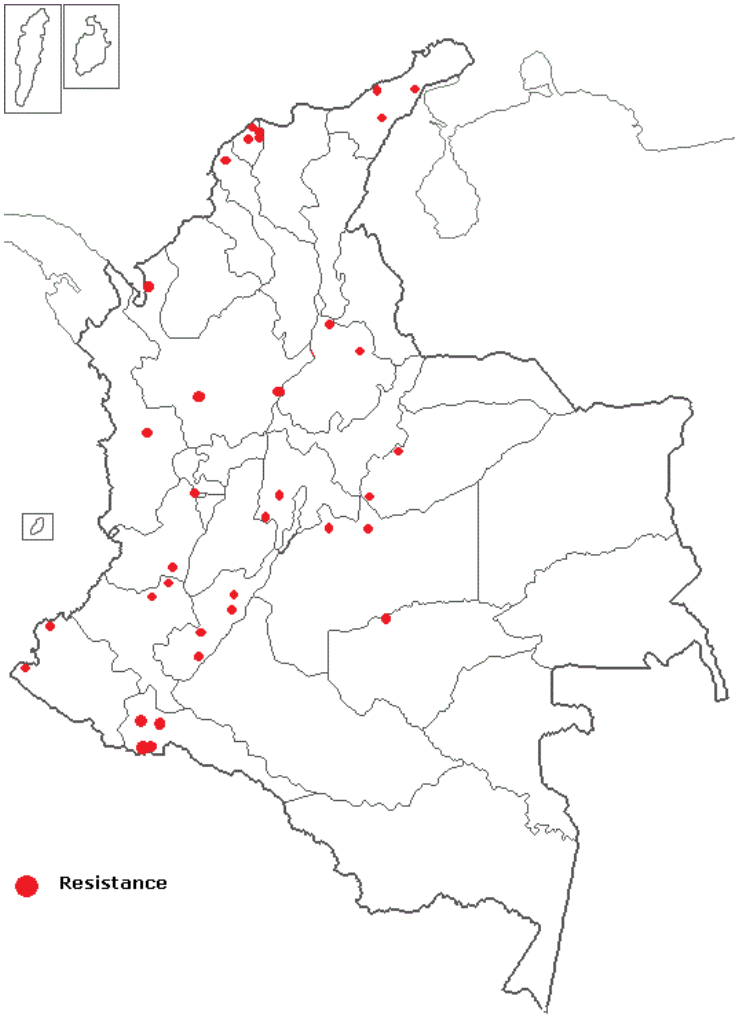


Fig. 4A. Susceptility status of *Aedes aegypti* populations to DDT in Colombia (CDC test).

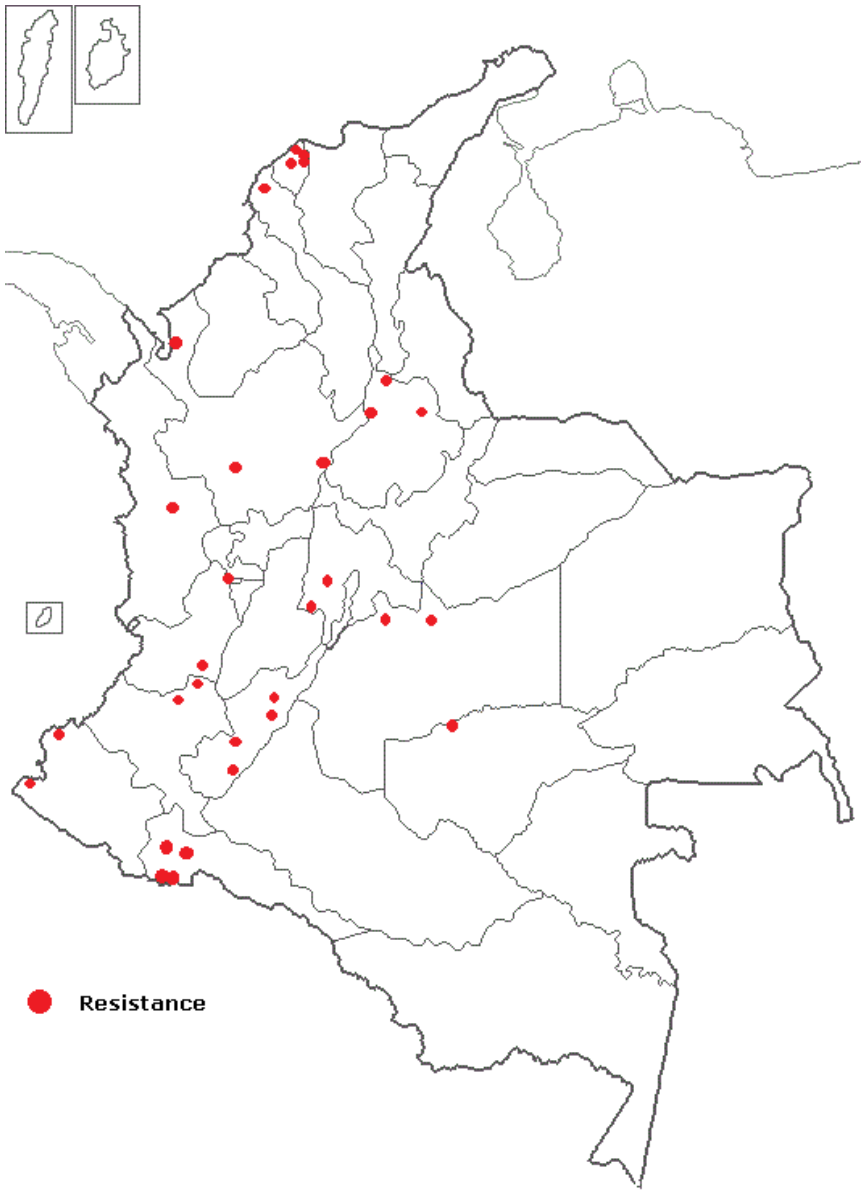


Fig. 4B. Susceptility status of *Aedes aegypti* populations to DDT in Colombia (OMS test).

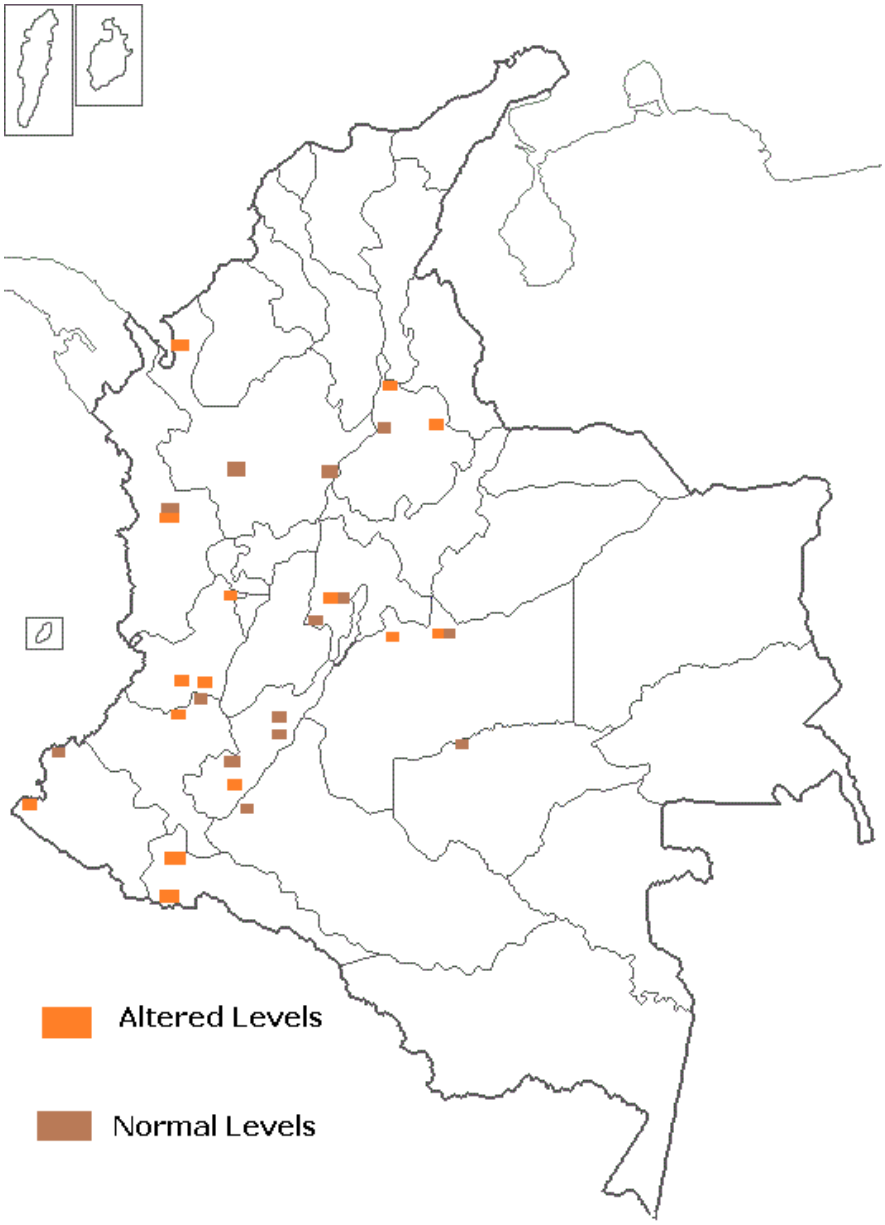


Fig. 5A. Non-specific esterases (NSE).

Fig. 5B. Mixed-function oxidases (MFO).

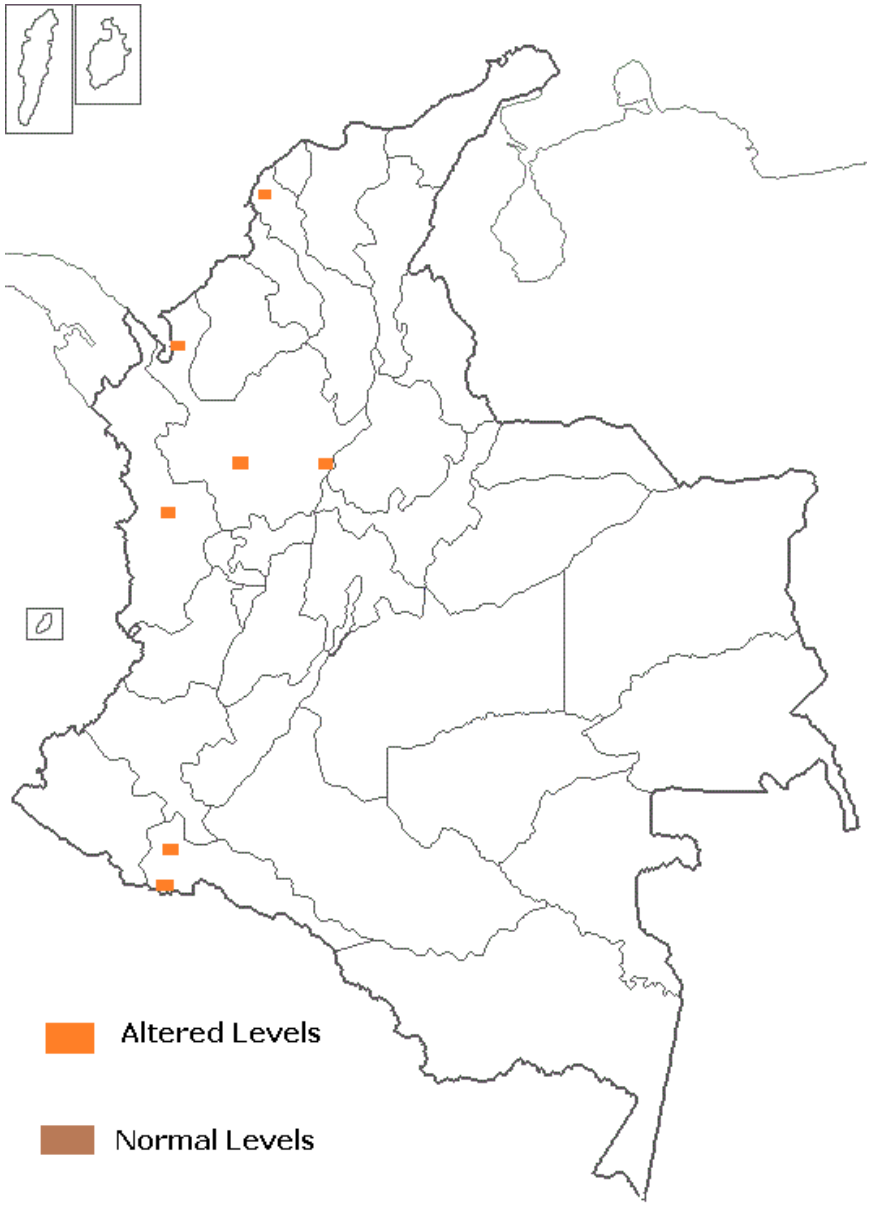
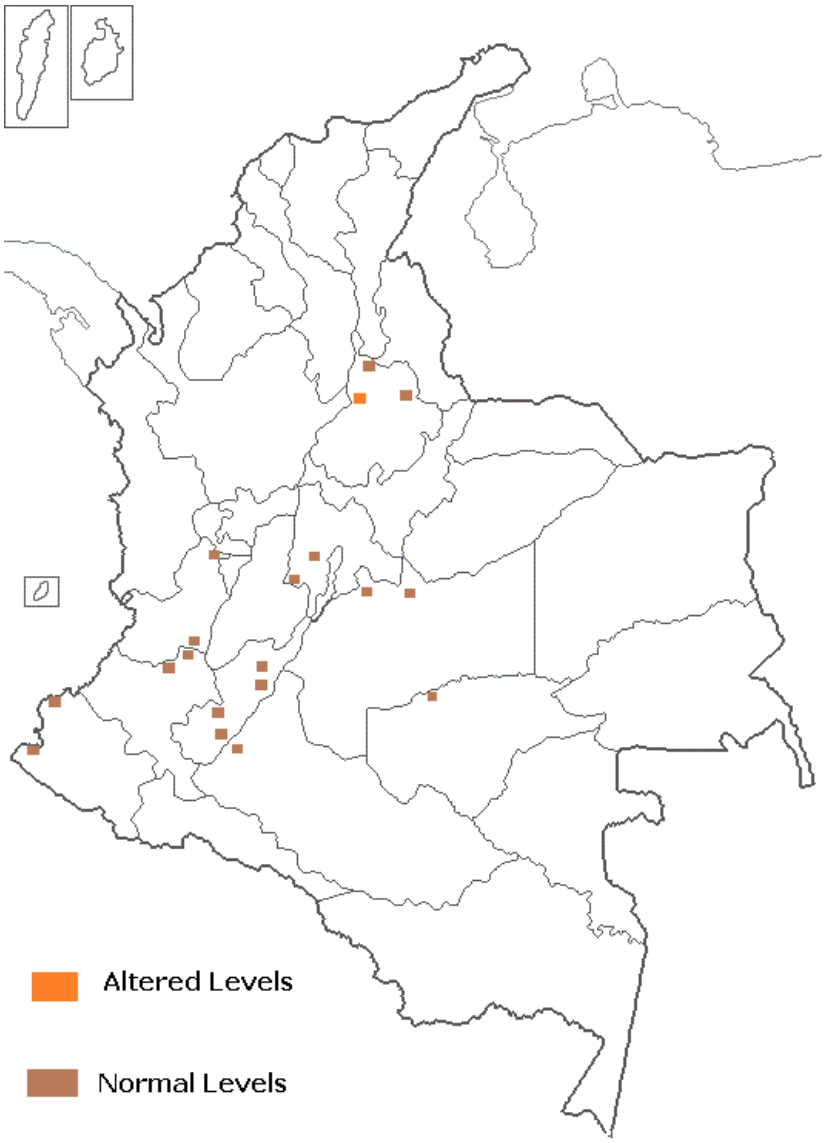


Fig. 5C. Glutathione-*S*-transferases (GST).



5D

Fig. 5. Biochemical mechanism of resistance in population of *Aedes aegypti* in Colombia: Non-specific esterases (NSE) (5A); Mixed-function oxidases (MFO) (5B); Glutathione-*S*-transferases (GST) (5C); acethylcholinesterase (AChE) (5D).

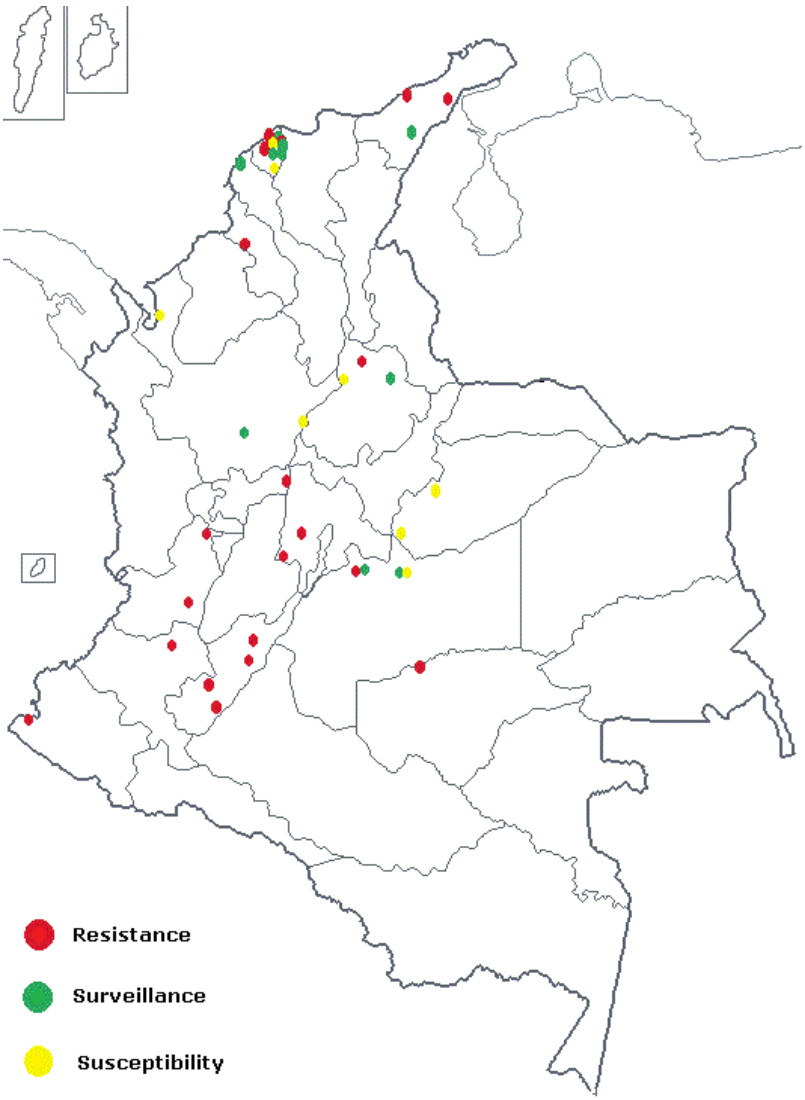


Fig. 6. Susceptility status of *Aedes aegypti* populations to Temephos in Colombia (OMS test).

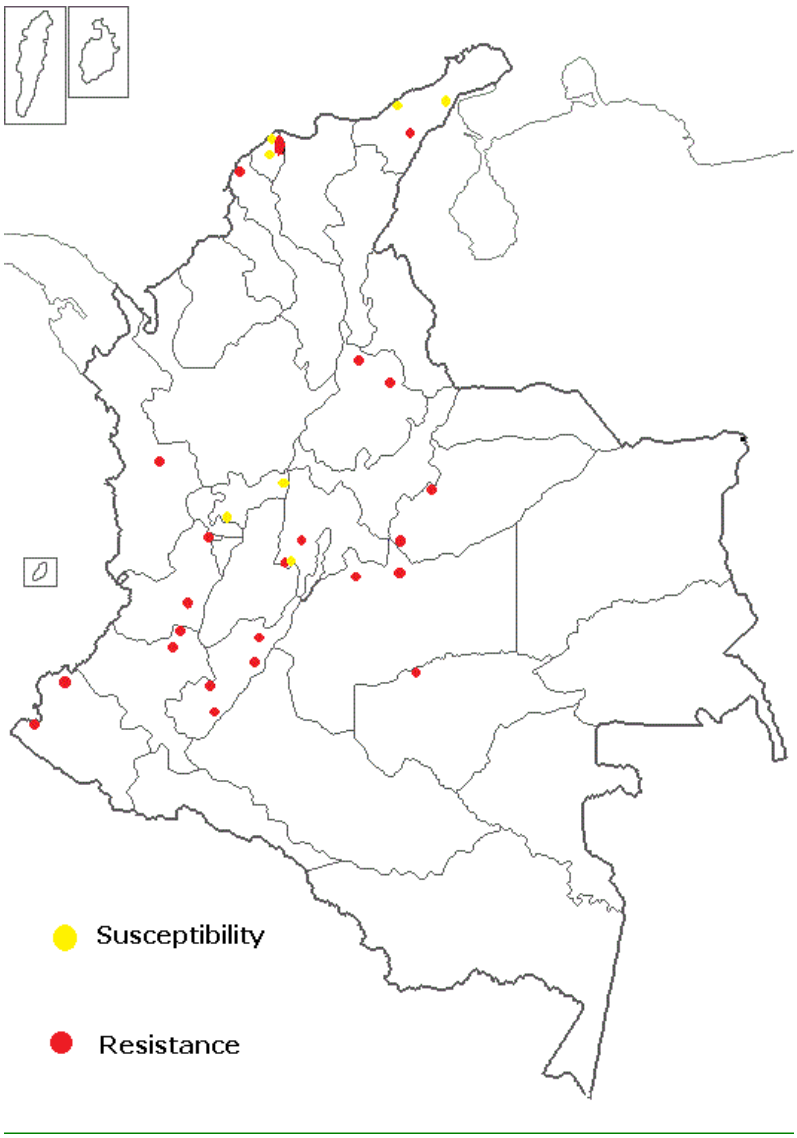


Fig. 7A. Susceptility status of *Aedes aegypti* populations to lambda-cyhalothrin in Colombia (CDC test).

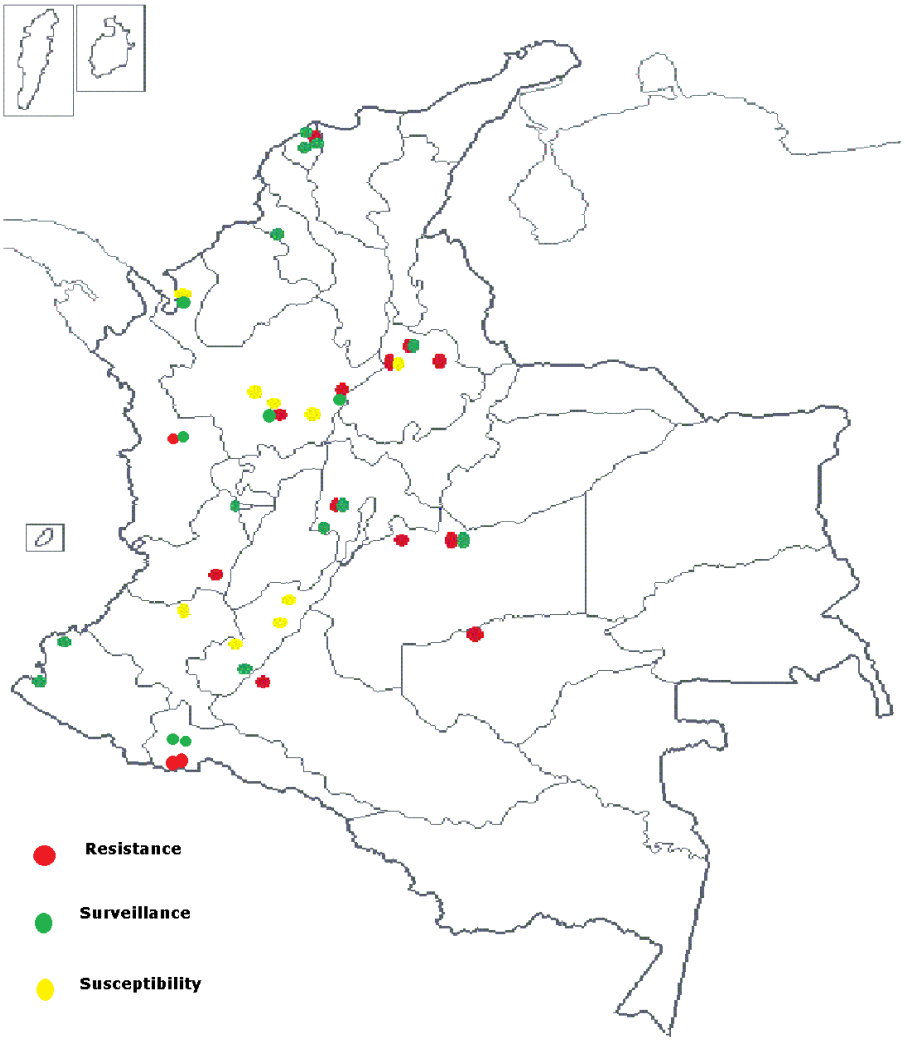


Fig. 7B. Susceptility status of *Aedes aegypti* populations to lambda-cyhalothrin in Colombia (OMS test).

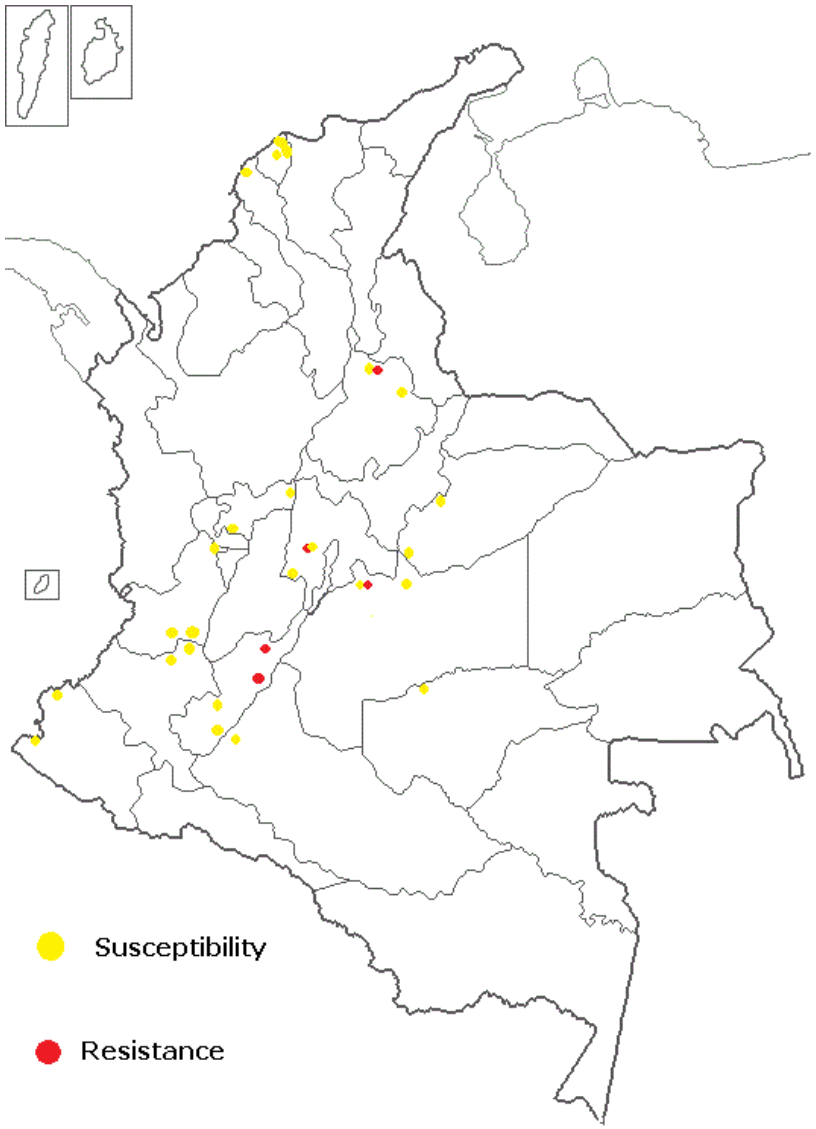


Fig. 7C. Susceptility status of *Aedes aegypti* populations to Deltamethrin in Colombia (CDC test).

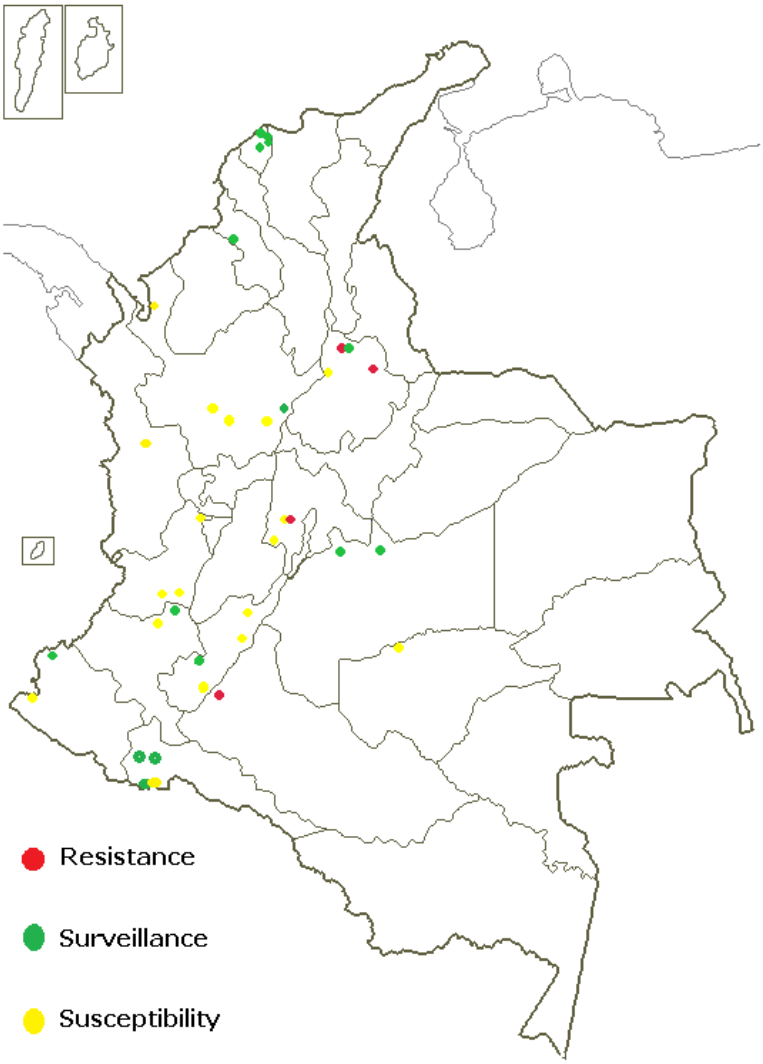


Fig. 7D. Susceptility status of *Aedes aegypti* populations to Deltamethrin in Colombia (OMS test).

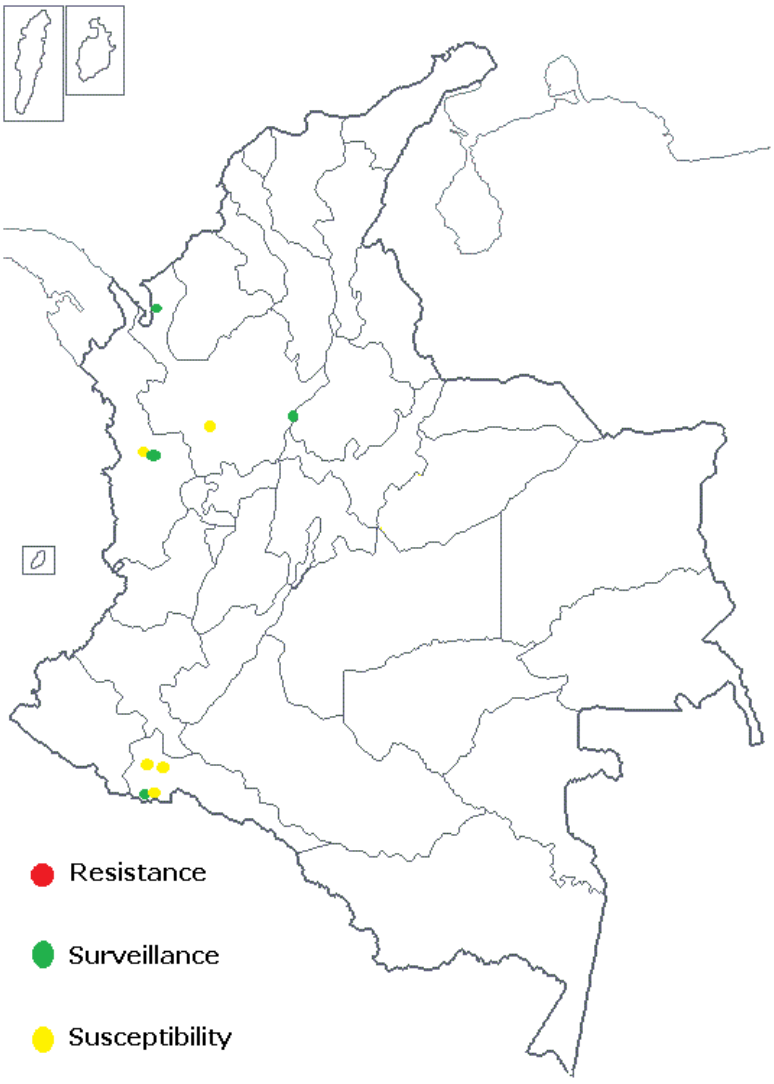


Fig. 7F. Susceptility status of *Aedes aegypti* populations to cyfluthrin in Colombia (OMS test).

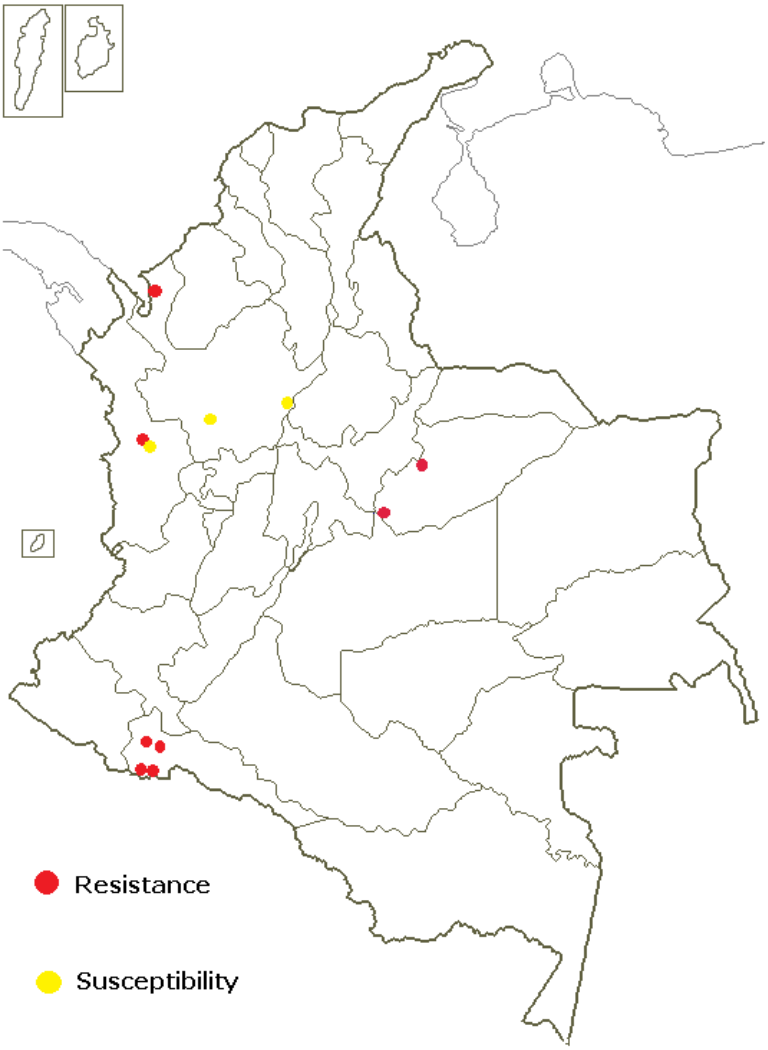


Fig. 7G. Susceptility status of *Aedes aegypti* populations to Permethrin in Colombia (CDC test).

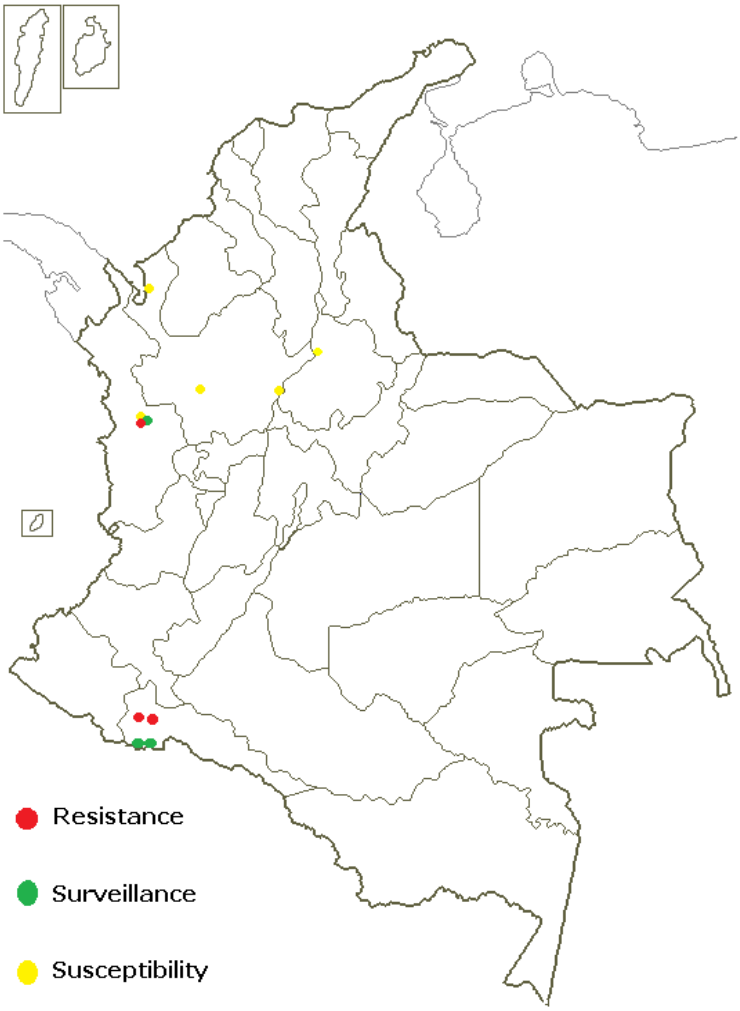


Fig. 7H. Susceptility status of *Aedes aegypti* populations to Permethrin in Colombia (OMS test).

Fig. 7I. Susceptility status of *Aedes aegypti* populations to etofenprox in Colombia (OMS test).

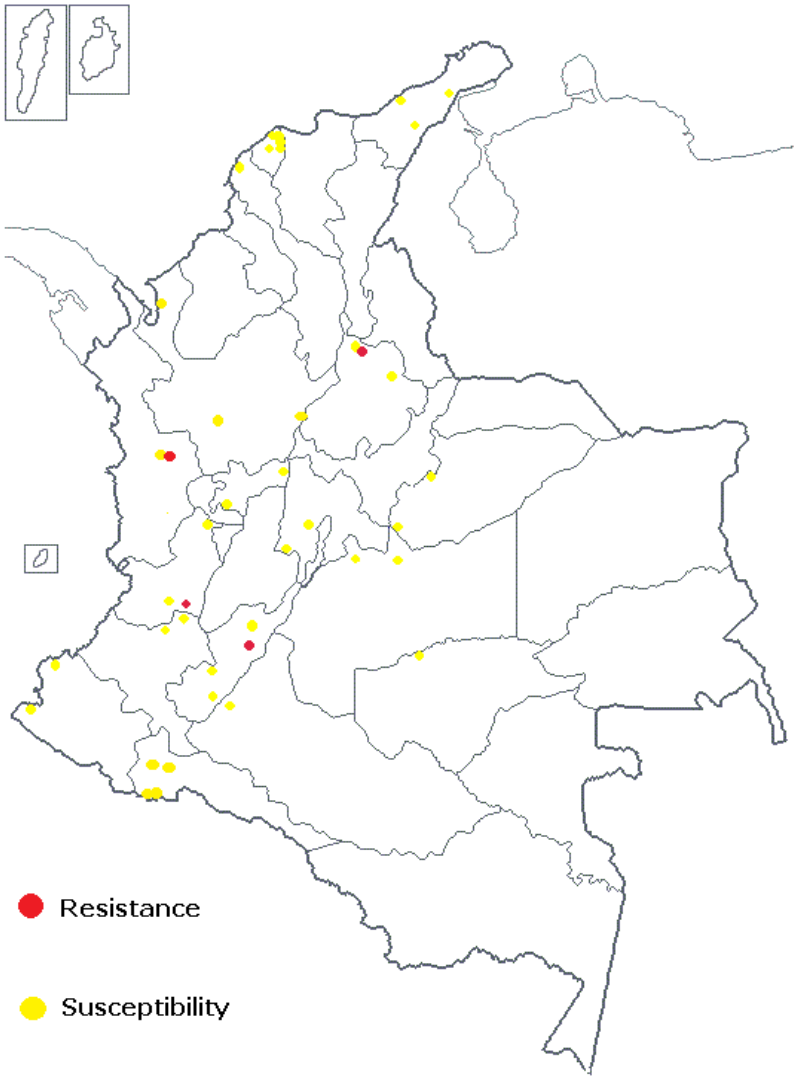


Fig. 8A. Susceptility status of *Aedes aegypti* populations to Malathion in Colombia (CDC test).

Fig. 8B. Susceptility status of *Aedes aegypti* populations to Malathion in Colombia (OMS test).

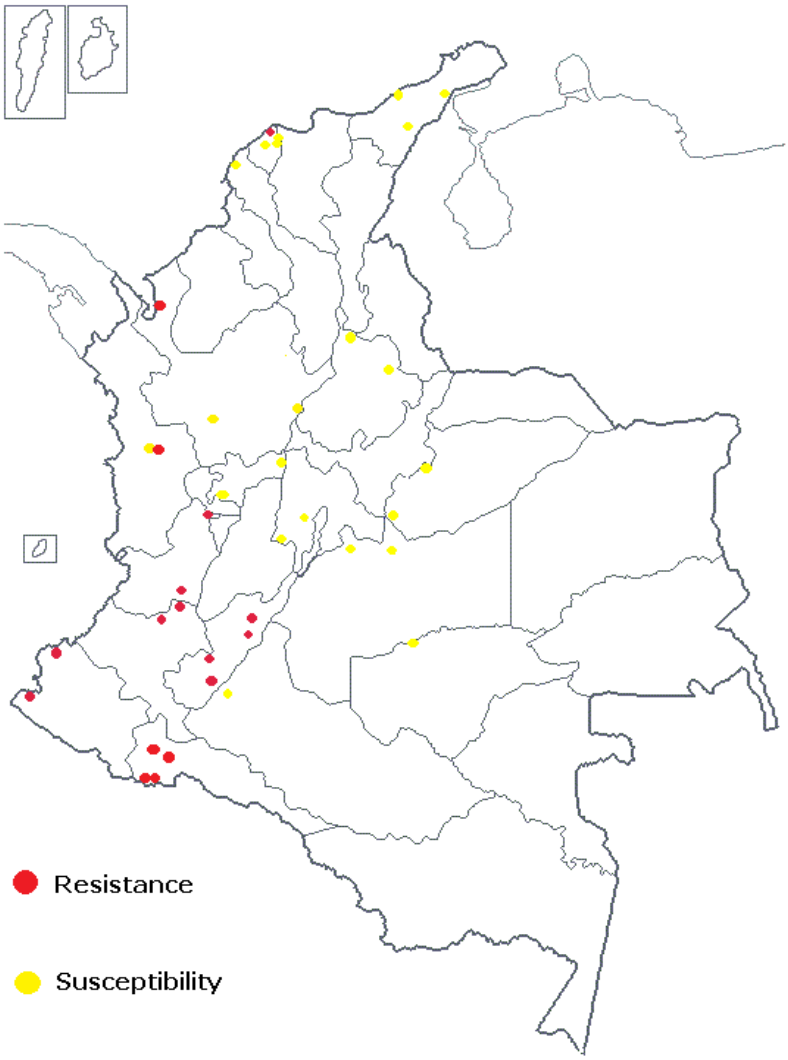


Fig. 8C. Susceptility status of *Aedes aegypti* populations to Fenitrothion in Colombia (CDC test).

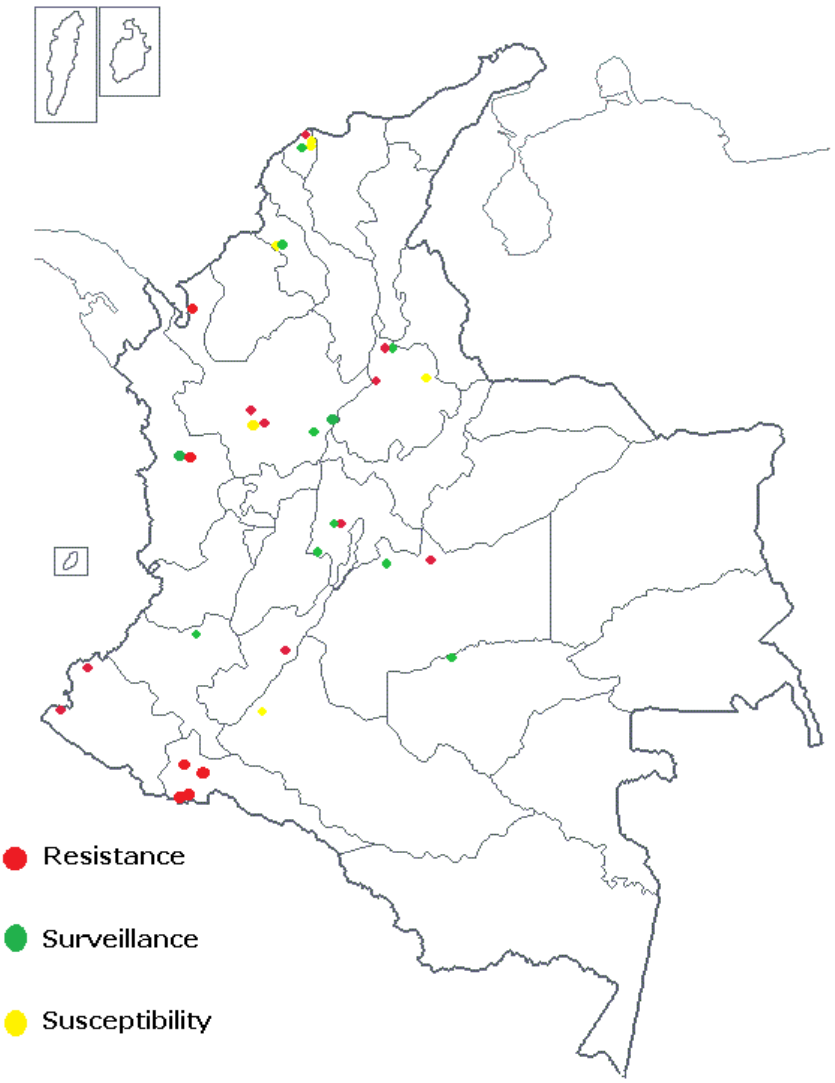


Fig. 8D. Susceptility status of *Aedes aegypti* populations to Fenitrothion in Colombia (OMS test).

Fig. 8E. Susceptility status of *Aedes aegypti* populations to pirimiphos methyl in Colombia (CDC test).

Fig. 8F. Susceptility status of *Aedes aegypti* populations to pirimiphos methyl in Colombia (OMS test).

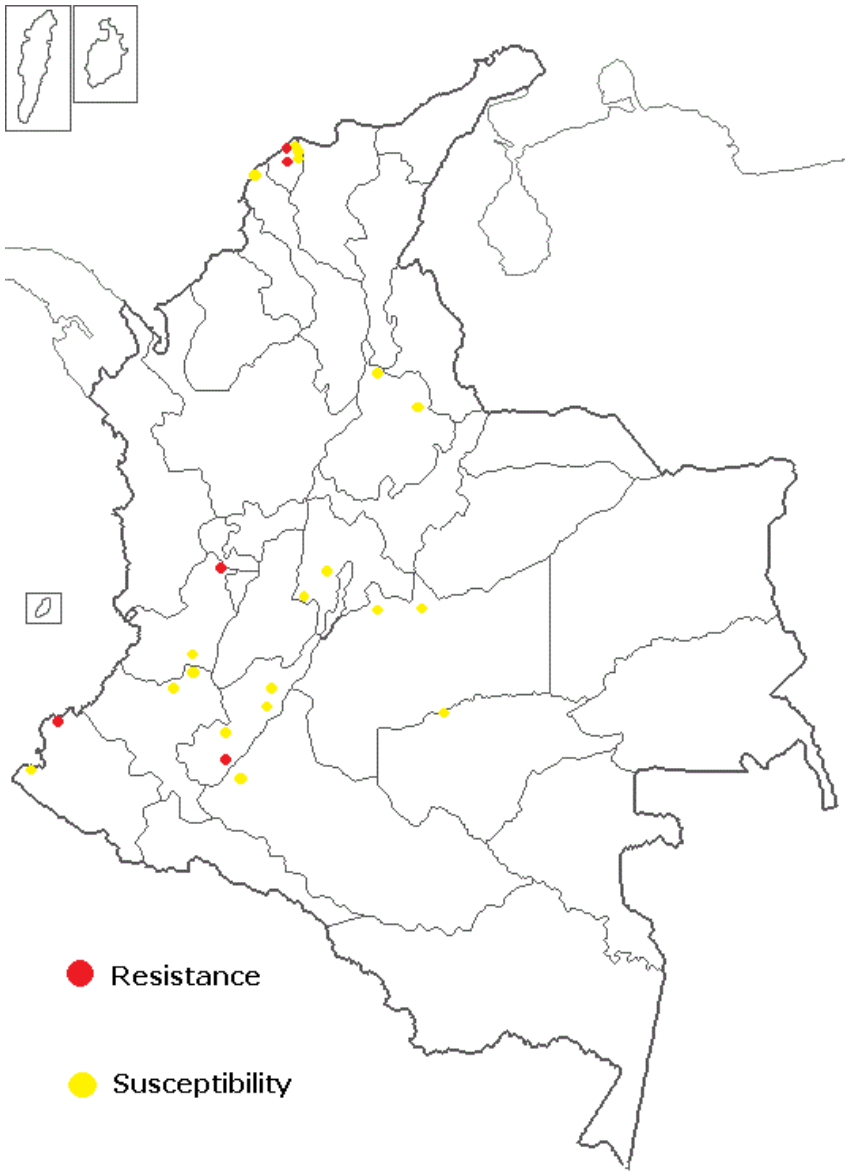


Fig. 9A. Susceptility status of *Aedes aegypti* populations to propoxur in Colombia (CDC test).

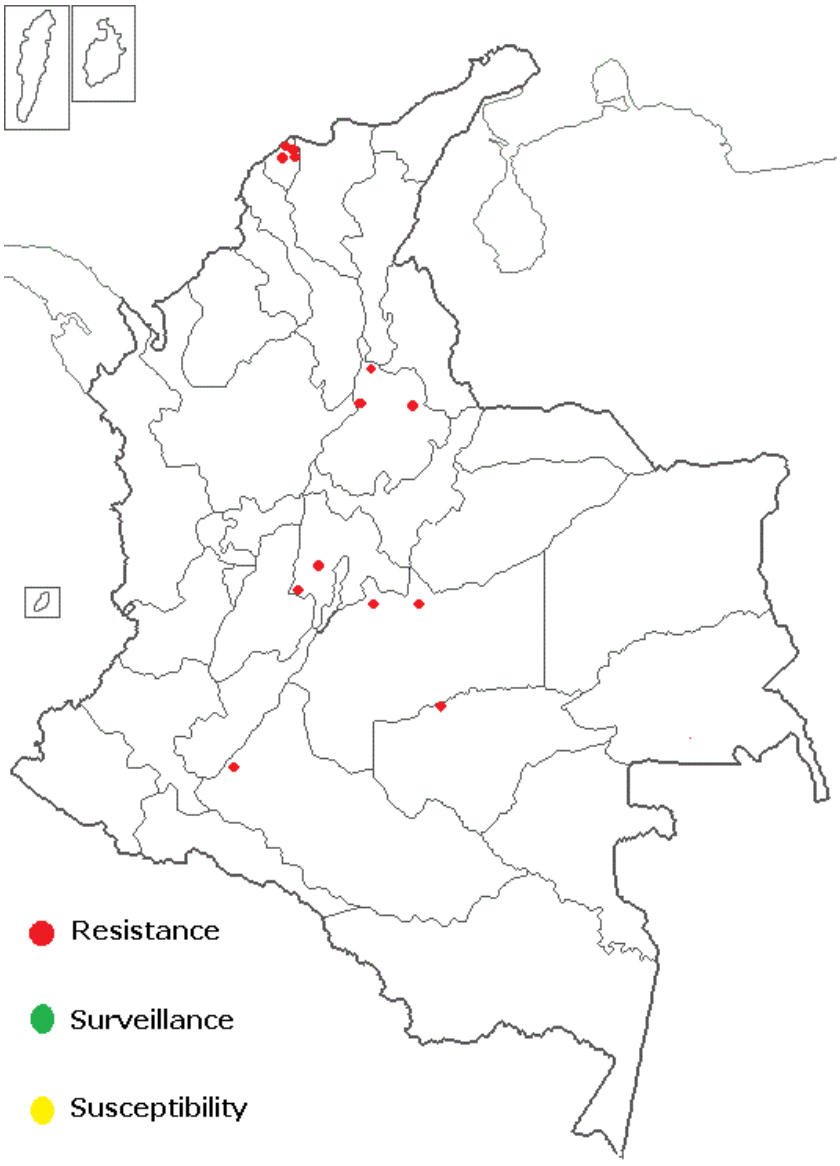


Fig. 9B. Susceptility status of *Aedes aegypti* populations to propoxur in Colombia (OMS test).

Fig. 10A. Susceptility status of *Aedes aegypti* populations to bendiocarb in Colombia (CDC test).

Fig. 10B. Susceptility status of *Aedes aegypti* populations to bendiocarb in Colombia (OMS test).

5. Conclusion

The selection pressure exert by insecticides for more than five decades on the populations of *A. aegypti* in Colombia, has generated widespread resistance to DDT and variability in susceptibility to phosphorated, carabamates and pyrethroids insecticides. As a possible cause of metabolic resistance to these insecticides, alteration in nonspecific esterase levels has been registered, mixed function oxidases and s-glutathione transferases. However, it is unknown whether there are alterations in target sites of action, especially in voltage-dependent sodium channel genes that explains the generalized resistance to DDT and most pyrethroids in the country.

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7. References

Albrieu-Llinás, G.; Seccacini, E.; Gardenal, CN. & Licastro, S. (2010). Current resistance status to temephos in *Aedes aegypti* from different regions of Argentina. *Mem Inst Oswaldo Cruz*, Vol.105, No.1, pp.113-116.

Álvarez, L.; Briceño, A. & Oviedo, M. (2006). Resistencia al temefos en poblaciones de *Aedes aegypti* (Diptera: Culicidae) del occidente de Venezuela. *Revista Colombiana de Entomología*, Vol.32, No.2, pp.172-5.

Anaya, Y.; Cochero, S.; Rey, G. & Santacoloma, L. (2007). Evaluación de la Susceptibilidad a insecticidas en *Aedes aegypti* (Diptera: Culicidae) capturados en Sincelejo. *Biomedica*, Vol.27, No.2, pp.257-8.

Anstead, JA.; Williamson, MS. & Denholm, I. (2005). Evidence for multiple origins of identical insecticide resistance mutations in the aphid Myzus persicae. *Insect Biochemistry and Molecular Biology*, Vol.35, pp.249–256.

Aparecida-Braga, I.; Pereira-Lima, J.; Da Silva-Soares, S. & Valle, D. (2004). *Aedes aegypti* resistance to temephos during 2001 in several municipalities in the states of Rio de Janeiro, Sergipe and Alagoas, Brazil. *Memórias do InstitutoOswaldo Cruz*, Vol.99, No.2, pp.199-203.

Ardila-Roldan, S. & Brochero, HL. (2010).Status of insecticide resistance in natural population from *Aedes aegypti* and CAP surveys of dengue vector in Casanare, Colombia. *Journal of American Mosquito Control Association,* Vol.26, No.3, pp.306-20.

Beserra, E.; Fernández, C.; De Quiroga, M. & De Castro, F. (2007). Resistance of *Aedes aegypti* (L.) (Diptera: Culicidae)populations to organophosphates temephos in the Paraíba State, Brazil. *Neotropical Entomology*, Vol.36, No.2, pp.303-7.

Bisset, JA.; Rodríguez, MM.; Molina, D.; Díaz, C. & Soca, LA. (2001). Esterasas elevadas como mecanismo de resistencia a insecticidas organofosforados en cepas de *Aedes aegypti*. *Revista Cubana de Medicina Tropical*, Vol.53, No.1, pp.37-43.

Bisset J. (2002).Uso correcto de insecticidas: control de la resistencia. *Revista Cubana de Medicina Tropical*, Vol.54, No.3, pp.202-219.

Bisset-Lazcano JA.; Rodríguez, MM.; San Martín, JL.; Romero, JE. & Montoya, R. (2009).Evaluación de la resistencia a insecticidas de una cepa de *Aedes aegypti* de El Salvador. *Revista Panamericana de Salud Publica*, Vol.26, No.3, pp.229-34.

Brogdon, W. & Mcallister, J. (1998).Simplification of adult mosquito bioassays through use of time mortality determinations in glass bottles. *Journal of the American Mosquito Control Association*, Vol.14, No2, pp.159-64.

Brengues, C.; Hawkes, NJ.; Chandre, F.; McCarroll, L.; Duchon, S. & Guillet, P. *et al*. (2003). Pyrethroid and DDT cross-resistance in *Aedes aegypti* is correlated with novel mutations in the voltagegated sodium channel gene. *Med Vet Entomol*., Vol.17, pp.87–94.

Brooke B. (2008). *kdr*: can a single mutation produce an entire insecticide resistance phenotype?. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, Vol.102, pp.524—525.

Brown, A. (1986). Insecticide resistance in mosquitoes: a pragmatic review. *Journal of American Mosquito Control Association*, Vol.2, pp.123-40.

Cadavid, JM.; Valderrama-Hernández, R.; Saenz-Osorio, O.; Quintero-Ruiz, B.; Rodríguez Ríos, C. & Contreras-Samper, A. (2008). Susceptibility of *Aedes aegypti* to insecticides in three high dengue transmission localities of Antioquia, Colombia *Journal of American Mosquito Control Association,* Vol.22, No.4, pp.748.

Chang, Ch.; Shen, WK.; Wang, TT.; Lin, YH.; Hsu, EL. & Dai, SM. (2009). A novel amino acid substitution in a voltage-gated sodium channel is associated with knockdown resistance to permethrin in *Aedes aegypti*. *Insect Biochemistry and Molecular Biology*, Vol.39, pp. 272–278

Chávez, J.; Vargas, J. & Vargas, F. (2005). Resistencia a deltametrina en dos poblaciones de *Aedes aegypti* (Diptera: Culicidae) del Perú. *Revista Peruana de Biología*, Vol.12, No1, pp.161-4.

Cui, F.; Weill, M.; Berthomieu, A.; Raymond, M. & Qiao, Ch. (2007). Characterization of novel esterases in insecticide-resistant mosquitoes. *Insect Biochemistry and Molecular Biology*,Vol.37, pp.1131–37.

Escobar JP. (2009). Políticas y orientaciones técnicas OPS/OMS para prevención y control del dengue y dengue hemorrágico. *Biomédica*, Vol.29, No1, pp.123-5.

Feyereisen R. (1999). Insect P450 enzymes. *Annu. Rev. Entomol*., Vol.44, pp.507-33.

Flores, A.; Albeldaño-Vásquez, W.; Fernández-Salas, I.; Badii, M.; Loaiza-Becerra, H.;

Ponce-Garcia, G.; Lozano-Fuentes, S.; Brogdon, W.; Black, W.; Beaty, B. (2005). Elevated á-esterase levels associated withpermethrin tolerance in *Aedes aegypti* (L.) from Baja California, México. *Pesticide Biochemistry and Physiology*, Vol.82, pp.66-78.

Fonseca, I. & Quiñones, M. (2005). Resistencia a insecticidas en mosquitos (Diptera: Culicidae): Mecanismos, detección y vigilancia en salud pública. *Revista Colombiana de Entomología*, Vol.31, No2, pp.107-115.

Fonseca I., Brant T.,Quiñones ML., Brogdonm, WG. (2006). Increased glutathione S transferases associated to DDT resistance in *Aedes aegypti* from Colombia. *Journal of American Mosquito Control Association,* Vol.22, No4, pp.738.

Fonseca, I.; Bolaños, D.; Gomez, W. & Quiñones, ML. (2007). Evaluación de la susceptibilidad de larvas de *Aedes aegypti* a insecticidas en el deprtamento del Antioquia. *Biomedica*, Vol.27, No.2, pp.176-7.

Fonseca-González, I. (2008). Estatus de la resistencia a insecticidas de los vectores primarios de malaria y dengue en Antioquia, Chocó, Norte de Santander y Putumayo, Colombia. [Tesis de doctorado]. Medellín: Universidad de Antioquia.

Fonseca, I. & Bolaños, D. (2009).Variación temporal en la susceptibilidad a malatión y Lambdacialotrina en *Aedes aegypti* (L) de Quibdo, Colombia. *Biomédica*, Vol.29, No.1, pp.218-9.

Fonseca-Gonzalez, I.; Quiñones, ML.; Lenhartc, A. & Brogdon, WG. (2011). Insecticide resistance status of *Aedes aegypti* (L.) from Colombia. *Pest Management Science*, Vol.67, No.4, pp.430-7.

Gómez-Camargo DE.; Maestre, RY.; Pisciotti, I.; Malambo, DI. & Gómez-Alegría, CJ. (2010). Insecticide susceptibility of *Aedes aegypti* in Cartagena (Colombia). *The American Journal of Tropical Medicine and Hygiene*, Vol.83, No.5, pp.298-9.

Hemingway, J. & Black IV, WC. (2007). A mutation in the voltage-gated sodium channel gene associated with pyrethroid resistance in Latin American *Aedes aegypti*. *Insect Molecular Biology*, Vol.16, No.6, pp.785-98.

Lumjuan, N.; Stevensona, BJ.; Prapanthadarab, L.; Somboonc, P.; Brophyd, PM.; Loftuse, BJ.; Severson f, DW. & Ransona, H. (2007). The *Aedes aegypti* glutathione transferase family. *Insect Biochemistry and Molecular Biology*, Vol.37, pp.1026-35.

Macoris, M.; Andrighetti, M.; Takaku, L.; Glasse, C.; Garbeloto, V. & Bracco, J. (2003). Resistance of *Aedes aegypti* from the state of Sao Paulo, Brazil to Organophosphates insecticidas. *Memórias do Instituto Oswaldo Cruz*, Vol.98, No.5, pp.703-8.

Maestre, R.; Rey, G.; De las Salas, J.; Vergara, C.; Santacoloma, L.; Goenaga, S. & Carrasquilla, MC. (2009). Susceptibilidad de *Aedes aegypti* (Diptera: Culicidae) a temefos en Atlántico-Colombia. *Revista Colombiana de Entomología,* Vol.35, No.2, pp.202-205.

Maestre-Serrano, R.; Goenaga, S.; Carrasquilla-Ferro MC.; Rey-Vega G.; Santacoloma-Baron L. & Vergara-Sánchez C. (2009). Estado de la susceptibilidad/resistencia a insecticidas en cuatro poblaciones de *Aedes aegypti* del departamento del Atlántico. *Biomedica*, Vol.29, No.1, pp.231-2.

Maestre, RY.; Florez, Z.; Cabrera, C.; Goenaga, S.; Gómez, D. & Gómez, C. (2010). Susceptibility status of *Aedes aegypti* to insecticides in La Guajira (Colombia). *The American Journal of Tropical Medicine and Hygiene,* Vol.83, No.5, pp.53.

Maestre, R.; Rey, G.; De las Salas, J.; Vergara, C.; Santacoloma, L.; Goenaga, S. & Carrasquilla, MC. (2010). Estado de la susceptibilidad de *Aedes aegypti* a insecticidas en Atlántico (Colombia). *Revista Colombiana de Entomología*,Vol.36, No.2, pp.242-248.

Maestre-Serrano, R.; Goenaga, S.; Flores, Z.; Cabrera C.; Gómez, D. & Gómez, C. (2010). *The Amercican Journal of Tropical Medicine and Hygiene* Vol.83, No.5, pp.53.

Martins, AJ.; Pereira-Lima, JB.; Peixoto, AA. & Valle, D. (2009). Frequency of Val1016Ile mutation in the voltage-gated sodium channel gene of *Aedes aegypti* Brazilian populations. *Tropical Medicine and International Health*, Vol.14, No.11, pp.1351-55.

Martinez-Torres, D.; Chandre, F.; Williamson, MS.; Darriet, F.; Berge, JB.; Devonshire, AL.; 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, pp.179–184.

Miller TA. 1988. Mechanisms of resistance to pyrethroid insecticides. *Parasitology Today*, Vol.4, pp.8-12.

Ocampo, C.; Salazar-Terreros, M.; Mina, N.; McAllister, J. & Brogdon, W. (2011). Insecticide Resistance status of *Aedes aegypti* in 10 localities of Colombia. *Acta Trop.*, Vol.118 No.1, pp.37-44.

Orjuela, L.; Herrera, M.; Quintana, N. & Quiñones, M. (2007). Estado de la susceptibilidad a insecticidas del mosquito *Aedes aegypti* en lo municipios de Chinchiná y La Dorada, Caldas. *Biomedica*, Vol.27, No.2, pp.176.

Pereira-da-Cunha, M.; Pereira-Lima JB.; Brogdon, WG.; Efrain-Moya G. & Valle D. (2005). Monitoring of resistance to the pyrethroid cypermethrin in Brazilian *Aedes aegypti* (Diptera: Culicidae) populations collected between 2001 and 2003. *Memorias do Instituto Oswaldo Cruz*, Vol.100, No.4, pp.441-44.

Pereira-Lima, E.; Da Oliveira-Filho, A.; Da Oliveira-Lima, J.; Ramos-Junior, A.; Da Goes-Cavalcanti, L. & Soares-Pontes, R. (2006). *Aedes aegypti* resistance to temephos in counties of Ceará State. *Revista da Sociedade Brasileira de Medicina Tropical,* Vol.39, No.3, pp.259-63.

Polson, KA.; Rawlins, SC.; Brogdon, WG. & Chadee, DD. (2010). Biochemical mechanisms involved in DDT and pyrethroid resistance in Trinidad and Tobago strains of *Aedes aegypti. The American Journal of tropical medicine and hygiene*, Vol.83, No.5, pp.51.

Poupardin, R.; Reynaud, S.; Strode, C.; Ranson, H.; Vontas, J. & David JP. (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, pp.540-51.

Quiñones, ML.; Suarez, MF. & Fleming, GA. (1987). Estado de la susceptibilidad al DDT de los principales vectores de malaria en Colombia y su implicación epidemiológica. *Biomédica*, Vol.7, pp.81-6.

Rawlins S. (1998). Distribución espacial e importancia de la resistencia a insecticidas de poblaciones de *Aedes aegypti* en el Caribe. *Revista Panamericana de Salud Pública,* Vol.4, No.4, pp.243-51.

Rodríguez, M.; Bisset, J.; Fernández, D. & Pérez, O. (2004). Resistencia a insecticidas en larvas y adultos de *Aedes aegypti*: prevalencia de esterasa A4 asociada con la resistencia a temefos. *Revista Cubana de Medicina Tropical*, Vol.56, No.1, pp.54-60.

Rodríguez, MM.; Bisset, JA. & Fernández, D. (2007). Determinación *in vivo* del papel de las enzimas esterasas y glutation transferasa en la resistencia a piretroides en *Aedes aegypti* (Diptera: Culicidae). *Revista Cubana de Medicina Tropical,* Vol.59, No.3, pp.209-12.

Rodríguez, M. (2008). Estudio de la resistencia a insecticidas en *Aedes aegypti* (Diptera: *Culicidae*). Tesis presentada al grado científico de Doctor en Ciencias de la Salud. Laboratorio de Toxicología y genética. Instituto de Medicina Tropical "Pedro Kourí". 128p.

Rojas, W.; Gonzalez, J.; Amud, MI.; Quiñones, ML. & Velez, ID. (2003). Evaluación de la susceptibilidad de Aedes aegypti del municipio de Barrancabermeja Santander a los insecticidas Malatión Fenitrotion, temefos, Lambdacihalotrina, deltametrina, permetrina, propoxur y DDT. *Biomédica,* Vol.23, No.1, pp.56

Rojas-Alvarez, DP. (2010). Comportamineto epidemiologico del dengue en Colombia, año 2009. *Informe quincenal Epidemiologico Nacional*, Vol.15, No.9, pp.129-37.

Saavedra-Rodriguez, K.; Urdaneta-Marquez, L.; Rajatileka, S.; Moulton, M.; Flores, AE.; Fernandez-Salas, I.; Bisset, J.; Rodriguez, M.; Mccall, PJ.; Donnelly, MJ.; Ranson, H.;

Salazar, M.; Carvajal, A.; Cuellar, ME.; Olaya, A.; Quiñones, J.; Velásquez, OL.; Viveros, A. & Ocampo, C. (2007). Resitencia a insecticidas en poblaciones de *Aedes aegypti* y *Anopheles* spp en los departamentos de Huila, valle Cauca y Nariño. *Biomédica*, Vol.27, No.2, pp.177.

Santacoloma, L. (2008). Estado de la susceptibilidad a insecticidas de poblaciones naturales de *Aedes aegypti* Linnaeus 1762 vector del dengue y *Anopheles darlingi* root 1926 vector primario de malaria (Diptera: Culicidae) en cinco departamentos de Colombia. [Tesis de maestría]. Bogotá: Universidad Nacional de Colombia.

Santacoloma-Varon, L.; Chavez-Cordoba, B. & Brochero, HL. (2010). Susceptibilidad de *Aedes aegypti* a DDT, Deltametrina y Lambdacialotrina en Colombia. *Revista Panamericana de Salud Publica*, Vol.27, No.1, pp.66-73.

http://www.todacolombia.com/geografia/ubicacion.html

Valderrama-Eslava, EI.; Gomzalez, R. & Jaramillo, GI. (2008). Evaluación de la susceptibilidad de *Aedes aegypti* (L) (Diptera: Culicidae) a un insecticida Organosfosforado y un piretroide en cuatro poblaciones del Valle del Cauca, mediante dos tipos de bioensayos. *Boletin del Museo de Entomología de la Universidad del Valle*, Vol.9, No.2, pp.1-11.

World Health Organization (WHO). (1957). Seventh report Expert Committee on insecticides *WHO Tech Report Ser*, Vol.125, pp.37.

World Health Organization (WHO). (1981a). Instructions for determining the susceptibility or resistance of adult mosquitoes to organoclorine, organophosphorus and carbamate insecticides. Establishment of the base-line. WHO/ VBC/81.805.

World Health Organization (WHO). (1981b). Instructions for determining the susceptibility or resistance of mosquitoes larvae to insecticide. Unpublished document.WHO/ VBC/81.807.

Yanola, J.; Somboon, P.; Walton, C.; Nachaiwieng, W. & Prapanthadara L. (2010). A novel F1552/C1552 point mutation in the *Aedes aegypti* voltage-gated sodium channel gene associated with permethrin resistance. *Pesticide Biochemistry and hysiology*, Vol.96, pp.127-13.

9

Behavioral Responses of Mosquitoes to Insecticides

Theeraphap Chareonviriyaphap
Department of Entomology, Faculty of Agriculture,
Kasetsart University, Bangkok,
Thailand

1. Introduction

Many people living in areas of the tropical and subtropical world are at serious risk of infection from a wide variety of vector-borne diseases, most notably malaria and dengue. Globally, approximately 50-100 million people are estimated to be at risk of infection with dengue viruses (the cause of dengue fever/dengue haemorrhagic fever) and between 100-300 million live in malaria endemic areas (World Health Organization [WHO], 2009). The viruses responsible for dengue are transmitted primarily by *Aedes aegypti*, a predominately urban, day-biting mosquito that often resides in and around human dwellings and preferentially feeds on humans (Gubler, 1997); whereas the 4 human malaria parasites (*Plasmodium*) are transmitted by a wide variety of *Anopheles* species (Service & Townson, 2002). Dengue vector has proven extremely resilient to control measures because of its close association and exploitation of domestic and peridomestic human settings (Reiter & Gubler, 1997). On the other hand, malaria vectors display a more diverse array of host seeking behaviors and preference, biting patterns and larval breeding habitats (Pates & Curtis, 2005; Sinka et al., 2011). Despite decades of extensive research, efficacious and commercially viable vaccines for these 2 important vector-borne diseases are not yet available. Therefore, the prevention and control of dengue and malaria remains dependent on various vector control strategies to reduce risk of transmission; in some instances this requires the use of various chemical insecticides as larvicides, space spray and indoor residual spray (IRS) applications, and use of insecticide-impregnated bed nets to control adult mosquito blood feeding (Roberts & Andre, 1994; WHO, 1999; Reiter & Gubler, 1997; Grieco et al., 2007).

Chemical insecticides, including organochlorines, organophosphates, carbamates, and synthetic pyrethroids, have long been used with great effect in public health vector control programs worldwide (Reiter & Gubler, 1997; Roberts & Andre, 1994; WHO, 1992). Although DDT used ceased in many countries several decades ago, the chemical has returned for use in malaria control IRS programs in Africa because of some its superior attributes (Roberts & Tren, 2010). The dramatic impact of DDT on mosquito populations in terms of both toxicity and behavior suppressing disease transmission is well known but in some instances the actual mechanisms at work remain unclear and poorly understood. Most studies on insecticides have placed attention exclusively on the direct toxicological (knockdown and killing) effects on mosquito populations; whereas far less research has focused on the

behavioral responses and outcomes as a result of chemical exposure (Roberts, 1993; Roberts et al., 1997; Chareonviriyaphap et al., 1997). Studies have shown that most chemical compounds influence insect locomotor (movement) behavior, often resulting in profound excitation and pre-mature movement away from treated surfaces or areas, one or more kinetic mechanisms resulting in so-called "avoidance behavior" (Kongmee et al., 2004; Miller et al., 2009). The term "excito-repellency" is often used to describe behavior that is stimulated by direct contact with a chemical that results in abnormal excitation (sometimes termed 'irritancy') and spatial repellency that results without the insect making physical contact with the chemical (Roberts et al., 1997). This chapter describes the various behavioral responses to insecticides in some important mosquito vectors of malaria (*Anopheles* species) (Table 1) and dengue (*Aedes aegypti*) (Table 2) and the tropical house mosquito, *Culex quinquefasciatus* (Table 3) using two types of laboratory systems and field assays using experimental huts.

2. History of test systems to study mosquito behaviors

Behavioral responses of mosquitoes to chemical compounds can be directly demonstrated by using various laboratory devices and field assay systems. For laboratory assays, many of the variations have been reviewed (Roberts et al., 1997). The WHO developed the first test box using plywoood in its construction attempting to access the excitation ("irritability") of exposed mosquitoes following physical contact with insecticides (WHO, 1970). This system was subsequently referred to as an "excito-repellency" test box (Rachou et al., 1963). Subsequently, the test system was further modified by other investigators interested in behavioral avoidance responses exposed to DDT and some of the early synthetic pyrethroids (Charlwood & Paraluppi, 1978; Roberts et al., 1984; Bondareva et al., 1986; Rozendaal et al., 1989; Quinones & Suarez, 1989; Ree & Loong, 1989). Years later, a lightproof test chamber was designed to study the irritant response of *Anopheles gambiae*, an importent malaria vector in Africa, to several chemical compounds (Evans, 1993). One key concern with all these test systems was associated with the technical difficulties of the test boxes for introducing and removing test specimens. Other concerns were controling for the various physiological conditions of wild-caught mosquitoes, and selecting the ideal range of concentrations for chemical compounds. In addition, no single or set of statistical methods for analysis of data has been fully accepted nor has any test system been specifically designed to truely discriminate between contact excitation and noncontact repellency responses (Roberts et al., 1997). An improved excito-repellency test device that was able to clearly differentiate between excitation and spatial repellency was developed and initially tested against several field populations of *Anopheles albimanus* from Central America (Roberts et al., 1997; Chareonviriyaphap et al., 1997). Unfortunately, this fixed prototype was cumbersome to handle and required considerable time for attaching the chemcial-treated test papers. Several years on, a more field-friendly test system was designed that was both collapsible and easily transportable (Chareonviriyaphap & Aum-Aong, 2000; Chareonviriyaphap et al., 2002) and was used to investigate the behavioral responses of various mosquito species and geographical populations from Thailand and a few populations from elsewhere in Asia (Chareonviriyaphap et al., 2001; Sungvornyothin et al., 2001; Kongmee et al., 2004; Pothikasikorn et al., 2005). Recently, a novel modular assay system was developed for mass screening of chemical actions; including contact irritancy, spatial repellency, or toxicity responses, on adult mosquitoes (Grieco et al., 2005). This

modular system is substantially reduced in size compared to the previous excito-repellency box and minimizes the treated surface area and therefore the amount of chemical required for testing. In field observations, numerous attempts have been made to determine behavioal responses of mosquitoes using specially constructed experimental huts (Smith, 1965; Roberts et al., 1984, 1987; Roberts & Alecrim, 1991; Rozendaal et al., 1989; Bangs, 1999; Grieco et al., 2000; Grieco et al., 2007; Polsomboon et al., 2008; Malaithong et al., 2010). Most experimental hut studies have been conducted to observe the behavior of *Anopheles* mosquitoes with far fewer studies on other genera. Grieco et al., (2007) successfully demonstrated all 3 chemical actions using *Ae. aegypti* as a model system. The results obtained from both laboratory and field studies can help faciliate the choice of the most effective chemical measures to control house-frequenting adult mosquitoes.

Two standard systems used in the laboratory, the excito-repellency box (ERB) and the high throughput screening system (HITSS), and the experimental hut in the field to help characterize the behavioral responses of mosquitoes to chemical compounds are discussed herein.

2.1 Excito-Repellency Box (ERB) test system

Given the complexities of insect behavioral research, the excito-repellency testing design and testing methodology along with methods of analyzing and interpreting test data, have yet to be universally accepted. However, tremendous improvement has occurred in recent years that effectively addressed a number of the previous drawbacks, opening up significance progress in behavioral studies involving innate responses to insecticides. The ERB test system (Fig.1) was developed to distinguish irritancy (Fig. 2) and repellency (Fig. 3) (Roberts, et al., 1997) and was first used to study the avoidance behavior of *Anopheles albimanus* to DDT and synthetic pyrethroids in Belize, Central America in which consistent and reliable results were generated measuring the escape behavior of female mosquitoes (Chareonviriyaphap et al., 1997). Over time, the initial system was modified into a collapsible test chamber with identical dimensions and operational attributes to help alleviate some of the previous handicaps (Chareonviriyaphap et al., 2002). This improved version has been used extensively in the evaluation of behavioral responses by laboratory and field mosquito populations in Thailand.

In 2006, a more field compatible device was designed with a substantial reduction in chamber size to minimize the amount of chemical and treated paper required, provide greater ease of test preparation and allow use of a small number of test specimens (15 vs. 25 females) compared to the larger version (Tanasinchayakul et al., 2006). Using this system, consistent and reliable results have been produced (Muenworn et al., 2006; Polsomboon et al., 2008; Thanispong et al., 2009; Mongkalagoon et al., 2009).

The system comprises of 4 outer stainless steel sheet metal walls (Fig. 1). Each wall is constructed with an aluminum sliding rib on each end and socket providing a surface for the test paper holder in the middle. The test paper holder consists of 2 sides: a sheet of fine mesh iron screen attached on one side and a panel to hold the test papers in place on top on the opposite side. There is a 0.8 cm gap between the test paper and screen barrier to prevent mosquitoes from making physical contact ('noncontact') on the treated paper surface during the repellency assay. The paper holder simply has to be inverted to provide the proper conditions to expose the paper in the contact test. On one end of the chamber a portal is made of overlapping sheets of dental dam and is used for placing mosquitoes inside the chamber and later for removing them after the exposure test period. A Plexiglas door serves

to seal the chamber and allow the investigator to view the exposure chamber before and after the actual test period. A stainless steel outer rear door cover is used to shut off all external light inside the chamber when the experiment is being performed.
Each tests consists of enclosing 15 female mosquitoes in each of 4 chambers lined with either insecticide-treated or untreated (control) papers. On the opposite side of the Plexiglas portal is a single exit portal for mosquitoes to escape to a receiving cage. At the beginning of a test, a 3-min rest period allows mosquitoes to adjust to test chamber conditions before the exit portal is opened to initiate the observation period (30 or 60 min depending on experiment). The numbers of mosquitoes escaping from the chamber into receiving cage are recorded at 1 min intervals until test completion.

Fig. 1. Excito-repellency test chamber showing side of exit portal (Roberts et al., 1997).

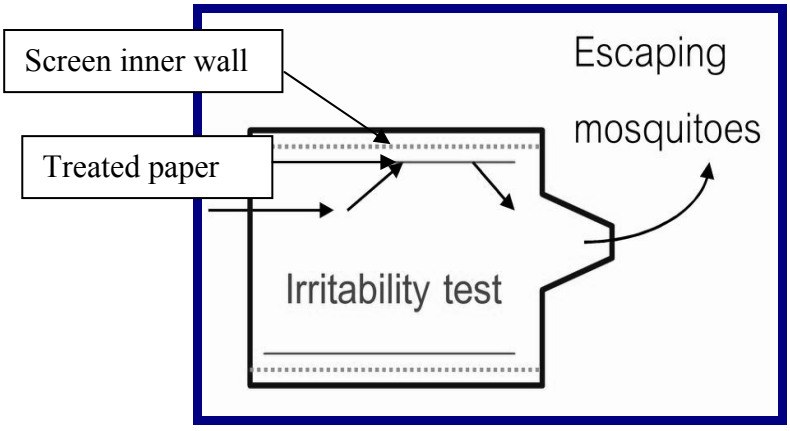

Fig. 2. Excito-repellency test chamber: Irritability test design (Roberts et al., 1997).

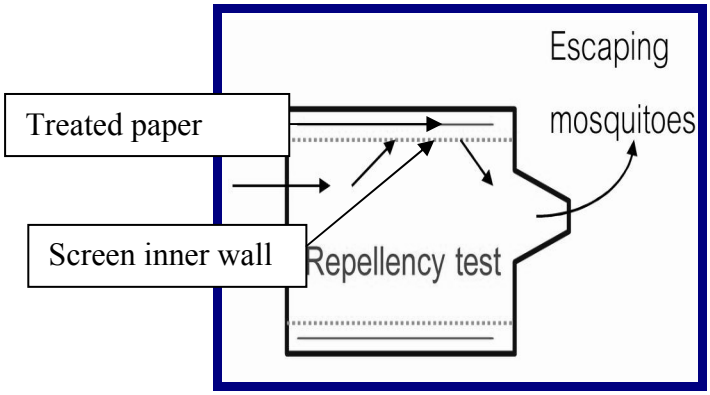

Fig. 3. Excito-repellency test chamber: Repellency test design (Roberts et al., 1997)

2.2 High Throughput Screening System (HITSS)

Even though an ERB test system has been found valuable to evaluate the innate behavioral responses of mosquito to chemical compounds, this system is relatively resource intensive requiring a comparatively large amount of chemical to be used for treating papers and having to use a large number of test mosquitoes. Moreover, a current ERB design is not conducive for mass screening the candidate chemical compounds. With the development of the HITSS assay (Grieco et al., 2005), a smaller amount of chemical and a lower number of mosquitoes is required per test. In addition, the HITSS can also be configured to test each of the three actions of insecticide compounds, namely contact irritancy assay (CIA) (Fig. 4), spatial repellency assay (SRA) (Fig. 5) and the toxicity assay (TOX) (Fig. 6). This modular system is made from a variety of durable materials including a thick, clear plastic cylinder, metal chambers, hard plastic end caps and a butterfly valve to control the opening and closing of the door. For the CIA, a single clear plastic cylinder is connected to a metal chamber lined with either treated or untreated netting material using a butterfly value placed in the open position (Fig. 4). For the SRA, an assay comprises a single clear plastic cylinder and 2 metal chambers on either end containing either treated or untreated (control) netting. The plastic chamber is positioned between the 2 metal chambers using a butterfly value as a linking system (Fig. 5). For the TOX assay, a single metal chamber is equipped with plastic end caps (solid cap on one side and tunnel cap on the other end). Netting material treated with either chemical active ingredient (treatment) or acetone carrier only (control) lines the inner chamber (Fig. 6). The HITSS has been standardized and used to evaluate the 3 behavioral responses of *Ae. aegypti* against DEET, Bayrepel®, and SS220 (Grieco et al., 2005, 2007). Recently, HITSS has been used to define the behavioral responses among six field populations of *Ae. aegypti* from Thailand against three synthetic pyrethroids (Thanispong et al., 2010). From this study, it was clearly shown that the HITSS assay is an effective and easy to use tool for distinguishing the three actions of chemicals and screening new compounds.

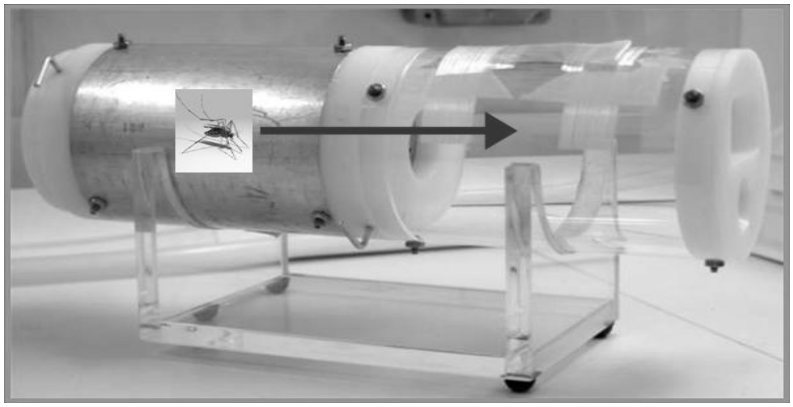

Fig. 4 High throughput screening system (HITSS): (CIA) Contact Irritant Assay (Grieco et al., 2005)

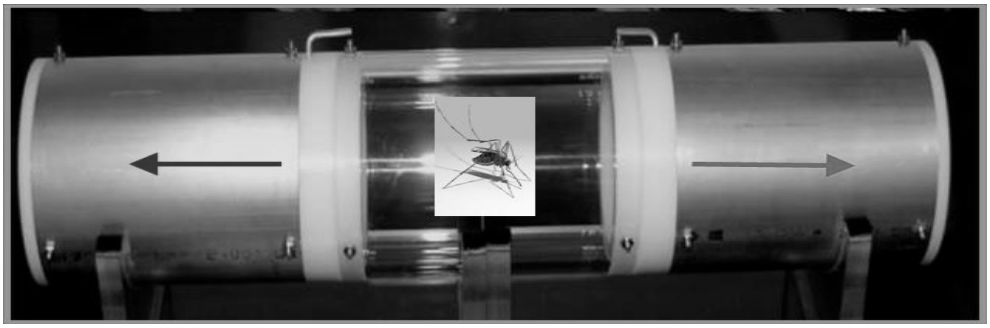

Fig. 5. High throughput screening system (HITSS): (SRA) Spatial Repellency Asay (Grieco et al., 2005)

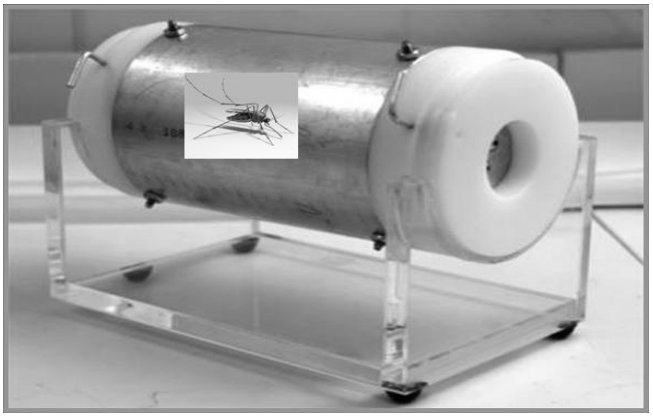

Fig. 6 High throughput screening system (HITSS): (TOX) Toxicity Assay (Grieco et al., 2005)

2.3 Field-based assay: experimental huts

To better understand the behavior of a mosquito exposed to a residual avoidance of under more natural, realistic conditions, field studies should be performed using experimental huts. So-called hut studies provide valuable information that can facilitate vector control operations by helping select the most appropraite and effective tool in combating disease vectors. Since the 1940s, most attention has been directed to the study of *Anopheles* mosquitoes, yet few investigations have been carried out to describe the innate behaviors of mosquitoes entering, resting, biting (blood feeding) and exiting human dwellings (Gahan & Lindquist, 1945; Giglioli, 1948; Smith, 1965; Roberts et al., 1984; Bangs, 1999; Grieco et al., 2000; Pates & Curtis, 2005; Roberts et al., 1997). Another important vector species, *Ae. aegypti*, has received even less attention using of experimental hut assays (Suwannachote et al., 2009). The discovery of using natural pyrethrum extract to prevent human-vector contact inside homes was well known prior to 1945 (Muirhead-Thomson, 1951) and the first report of behavioral responses of malaria vectors to DDT was documented in 1947 (Kennedy, 1947, Brown, 1983). Strong behavioral responses to DDT were progressively reported up until 1975 (Muirhead-Thomson, 1960; Elliott & de Zulueta, 1975; Brown, 1983). For example, de Zulueta and Cullen (1963) observed significant reductions in the numbers of biting mosqutioes on human indoors and resting on walls in houses sprayed with DDT. However, there was no clear explanation on how (mechanism) DDT functioned by either repelling or preventing mosquitoes from locating host stimuli inside sprayed houses. A significant scientific observation on behavioral avoidance of *Anopheles ganbiae* was carried out in the mid 1960s (Smith & Webley, 1968) wherein 2 huts, 1 control and one treated with DDT were equipped with verandah traps and gas chromatography was used to evaluate the subsequent response of mosquitoes. A hut treated with DDT was observed to prevent between 60 and 70% of *An. gambiae* from normally entering indoors as an outflow of DDT was continuously decreasing. Additionally, those females that did enter the sprayed hut became stimulated (irritated) and escaped the hut much quicker than those mosqutioes present in the unsprayed hut. Another study comparing biting patterns of *Anopheles minimus* in Thailand showed a 71.5% decline in attempted blood feeding inside the DDT treated hut and a 42.8% reduction in blood feeding success in a deltamethrin treated hut (Polsomboon et al., 2008). Using the same huts, observing human landing patterns of *Anopheles dirus* complex found that the relative risk (odds) of female mosquitoes entering and attempting to feed were half the number when exposed to DDT compared with the deltamethrin treated hut (Malaithong et al., 2010).

Of the possible responses a mosquto exposed to a chemcial can preform, spatial repellency is prehaps the most interesting and important. Observing true repellency presents problems of accurate messurement in the natural field situation. One of classic methods is to use the experimental huts wherein 2 experimental huts, 1 untreated control and 1 treated with an active ingredient is fitted with entrance (measure of repellency) and exit (irritancy) traps as illustrated in (Figs. 7-9). A landmark study was conducted by Grieco et al., (2007) on the movement patterns of *Ae. aegpyti* into and out of the experimental huts alternatively equipped with entrance (Fig. 8) and exit traps (Fig. 9) to describe the 3 chemical actions and mosquito responses as previously proposed in the mathematical framework for understanding the impact and relevance of repellency, irritability and toxicity on mosquito populations and disease transmission (Roberts et al. 2000). This study clearly showed that the 3 actions described and help to validate the model of actions. It was concluded that contact irritancy is the predominant action of synthetic pyrethroids, whereas spatial

repellency is the primary action of DDT. Dieldrin, a once commonly used cyclodiene compound, exhibited primarily a toxic action only.

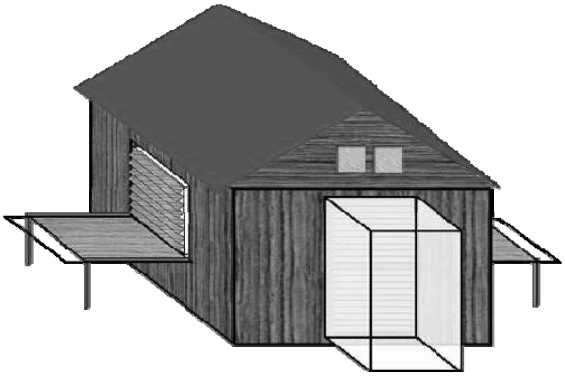

Fig. 7 Experimental hut with traps attached to exterior walls.

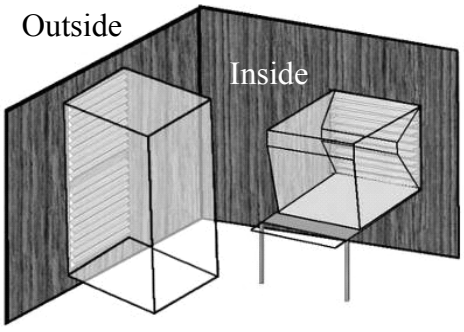

Fig. 8 Experimental hut with entrance traps attached to interior walls.

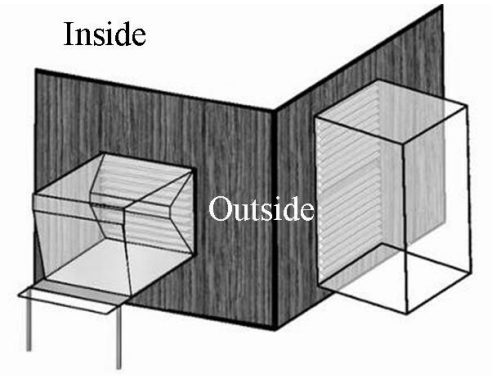

Fig. 9. Experimental hut with exit traps attached to exterior walls.

3. Behavioral responses

To facilitate the study of behavioral responses of mosquitoes to chemical compounds, several test systems have been developed (Roberts et al., 1997; Chareonviriyaphap et al., 2001). The following is a brief review of important historical findings and more recent work from studies in Thialand on behavioral responses of mosquitoes to DDT and various synthetic pyrethroids commonly used in vector control.

3.1 Behaviroral responses to DDT

Some agricultural and medically important insects, including vectors of malaria, demonstrate what has been termed "behavioral resistance" to DDT (Lockwood et al., 1984). However, the term "behavioral avoidance" as an innate response rather than a permanent genetic shift in behavior is preferred since the development of behavioral changes due to insecticide selective pressure in nature has not been adequately documented (Muirhead-Thomson, 1960). The behavioral responses of mosquitoes have been investigated using either specially constructed experimental huts and/or ERB test system. The first study on the irritant effect of DDT residual deposits was conducted on *Anopheles quadrimaculatus* where females were found to be irritated after short contact with treated surfaces with many quickly escaping the DDT treated house without taking a blood meal (Gahan & Lindquist, 1945). Subsequent observations found that *An. quadrimaculatus* had received a lethal dose and perished within 24 hours (Metcalf et al., 1945). Unfortunately, these studies made the observations without having control (untreated houses) for comparison. Moreover, the high mortality seen with *An. quadrimaculatus* may have been caused by further contacts with toxic active ingredients while attempting to leave a treated house through a small outlet (Muirhead-Thomson, 1960). In the studies with *Anopheles albimanus* in Panama, Trapido (1954) concluded that wild-caught mosquitoes lacking re-exposure to DDT for a long period of time, showed the same susceptibility levels to DDT as those from a laboratory colony with no a history of previous exposure. Malaria vectors in some countries (e.g., Brazil, Thailand) have never developed resistance to DDT (Roberts et al., 1984; Chareonviriyaphap et al., 2001), suggesting that the particular mosquito population possibly avoids making direct physical contact with the chemical, thereby precluding any selection for resistance. Table 1 lists *Anopheles* species tested and levels of behavioral responses to DDT and synthetic pyrethroids. DDT was found to be a strong contact irritant and more moderately as a repellent among 3 test populations of *An. albimanus* and that most specimens that quickly escaped DDT exposure survived (Chareonviriyaphap et al., 1997). This finding is in agreement with the results of the field studies by Roberts and Alecrim (1991) who reported a strong repellent action of DDT with *Anopheles darlingi* in a sprayed house. In a similar study, both irritancy and repellency escape responses were observed in 2 populations of *Anopheles minimus* with a major action of DDT being contact excitation (Chareonviriyaphap et al., 2001). Another study was made on the 2 complex species within the Minimus Subgroup; *An. minimus* (species A) and *Anopheles harrisoni* (species C). DDT produced a rapid and striking irritancy response in both species. Additionally, repellency was more pronounced with DDT on *An. minimus* but was seen to be much weaker with *An. harrisoni* (Pothikasikorn et al. 2005). With *Ae. aegypti*, results demonstrated that the higher the degree of physiological resistance to DDT, the greater the apparent suppression of both contact irritant and noncontact repellency responses, yet avoidance behavior was still a significant event (Thanispong et al., 2009).

Species	Strain	Permethrin		Deltamethrin		Cypermethrin		λ-cyhalothrin		Bifenthrin		DDT		References
		I	*R*	*I*	*R*	*I*	*R*			*I*	*R*	*I*	*R*	
An. albimanus	Lab	+	-	+	-							+	-	Chareonviriyaphap et al., 1997
An. albimanus	Field	++++	-	++++	-							++++	++	Chareonviriyaphap et al., 1997
An. albimanus	Field	++++	-	++++	-							++++	++	Chareonviriyaphap et al., 1997
An. albimanus	Field	++++	-	++++	-							++++	++	Chareonviriyaphap et al., 1997
An. minimus	Lab			++++	+			+++	-			+++	+	Chareonviriyaphap et al., 2001
An. minimus	Field			++	+			++++	-			+++	+	Chareonviriyaphap et al., 2001
An. minimus	Lab			++++	+++									Chareonviriyaphap et al., 2004
An. minimus	Lab			++++	++									Chareonviriyaphap et al., 2004
An. dirus	Lab			+++	+++									Chareonviriyaphap et al., 2004
An. maculatus	Field			++++	+									Chareonviriyaphap et al., 2004
An swadwongporni	Field			++++	++									Chareonviriyaphap et al. 2004
An. dirus	Field			+++	++									Chareonviriyaphap et al. 2004
An. minimus	Field			++++	++			++++	-			++++	+++	Potikasikorn et al., 2005
An. harrisoni	Field			++++	-			++++	-			++++	-	Potikasikorn et al., 2005
An. maculatus	Field	+++	-									+	-	Muenworm et al., 2006
An swadwongporni	Field	+++	-									+++	-	Muenworm et al., 2006
An. minimus	Field					++	+							Pothikasikorn et al., 2007
An. minimus	Field									+	-			Tisgratog et al., 2011
An.harrisoni	Field									+++	+			Tisgratog et al., 2011

I: Irritancy (Excitation and movement away from the source following direct contact with chemical stimulant)
R: Repellency (noncontact, spatial detection of chemical that results in movement away from the source)
++++: 81-100% escaped from treated chamber
+++: 61-79% escaped from treated chamber
++: 41-59% escaped from treated chamber
+: 21-40% escaped from treated chamber
-: ≤ 20% escaped from treated chamber but statistically significant ($P < 0.05$) from the matched control

Table 1. Degree of behavioral responses of female *Anopheles* species to synthetic pyrethroids and DDT at an operational field dose (mg/cm²).

3.2 Behavioral response to synthetic pyrethroids

A number of synthetic pyrethroids, i.e. allethrin, deltamethrin, permethrin, cypermethrin, alpha-cypermethrin, cyfluthrin among others, are commonly used by home owners, private business and government sectors to control both household mosquitoes and those as important vectors. These pyrethroids have found to exhibit a moderate to strong repellent effect for many agricultural and medically important insects (Lockwood et al., 1984) and were observed to cause mosquitoes to move away ('avoidance') from sprayed areas (Miller, 1990; Lindsay et al., 1991). The extensive and continuing use of pyrethroids should be a major stimulus to intensify observations on the significance of pyrethroid-induced avoidance behavior in mosquito vectors and other arthropods. Given the role of indoor residual spraying of homes as a means of controlling malaria transmission, the precise role of excitation and repellent actions of pyrethriods should be well defined for specific malaria vectors prior to beginning any large scale control program. Following the refinement of the ERB test system allowing separation of the 2 types of primary behavioral actions (Roberts et al., 1997), a series of important findings on excito-repellency behavior in *Anopheles* mosquitoes have been subsequently reported (Chareonviriyaphap et al., 1997, 2001, 2004; Pothikasikorn et al., 2005; Muenworn et al., 2006; Pothikasikorn et al., 2007). In general, synthetic pyrethroids produce much stronger irritant responses in *Anopheles* compared to repellency action (Table 1). For example, lambda-cyhalothrin and deltamethrin act as strong irritants on test populations of *An. minimus* complex mosquitoes while showing relatively weak repellency action (Chareonviriyaphap et al., 2001). Pothikasikorn et al. (2005) confirmed that Minimus complex species, *An. minimus* and *An. harrisoni* show a rapid irritancy to lambda-cyhalothrin and deltamethrin. Chareonviriyaphap et al., (2004) produced an extensive study to define the excito-repellency action of deltamethrin on 4 *Anopheles* species, all representing important malaria vectors in Thailand. Again, the findings demonstrated that deltamethrin produced a pronounced irritancy action compared to a much weaker repellency effect. Although repellency was less profound than contact excitation, the escape responses were statistically significant compared to the matched controls. A number of *Ae. aegypti* populations have been tested against a series of synthetic pyrethroids (deltamethrin, permethrin, alphacypermethrin, cyphenothrin, d-tetramethrin and tetramethrin) (Table 2). In general, all test populations of *Ae. aegypti* populations exhibit moderate to strong irritancy as compared with repellency (Grieco et al., 2005; Chareonviriyaphap et al., 2006; Paeporn et al., 2007; Thanispong et al., 2009, 2010). In addition, a few populations of *Culex quinqaefacsciatus* have been tested against the 3 principal classes of insecticides used in vector control; pyrethroids (deltamethrin), organophosphates (fenitrothion) and carbamates (propoxur) (Table 3). Striking differences in behavioral responses were seen between populations and active ingredients. Greater contact escape action was observed in a long-established colony exposed to deltamethrin and fenitrothion compared to two recent field populations (Sathantriphop et al., 2006).

To summarize, the behavioral responses to insecticides by mosquitoes are important components of a chemical's overall effectiveness in reducing human-vector contact and transmision of disease. To date, there is no convincing example of behavioral resistance in mosquito species to insecticides, rather all evidence indicates actions on the part of exposed mosquitoes are part of an innate behaviral repertoire. Behavioral response can be split into 2 distinct categories, stimulus-dependent and stimulus-independent actions (Georghiou, 1972). A stimulus-dependent response requires sensory stimulation of the insect in order for an avoidance action to proceed. In general, this form of avoidance enables the insect to

Strains	Deltamethrin		Permethrin		α-cypermethrin		Cyphenothrin		d-tetramethrin		Tetramethrin		DDT		References
	I	R	I	R	I	R	I	R	I	R	I	R	I	R	
Field-R	++	-													Kongmee et al., 2004
Field-R	+	-													Kongmee et al., 2004
Field-R	+	-													Kongmee et al., 2004
Field-R	++++	-													Kongmee et al., 2004
Field-R	++	-													Kongmee et al., 2004
Field-S	+++	-													Kongmee et al., 2004
Lab-S	++	-													Kongmee et al., 2004
Lab-S	++	-													Kongmee et al., 2004
Lab-S	++	-													Kongmee et al., 2004
Field-RR			+	-											Paeporn et al., 2007
Field-RR			-	-											Paeporn et al., 2007
Field					++++	-							++	-	Thanispong et al., 2009
Field-S					+++	-							-	-	Thanispong et al., 2009
Lab-S					++++	++							++	++	Thanispong et al., 2009
Field-S	+++	+					++++	-	+++	+	++	+			Mongkalagoon et al., 2009
Field-S	++++	-					++++	+	+++	+	+++	-			Mongkalagoon et al., 2009
Field-S	++++	+					++++	+	+++	-	+++	+			Mongkalagoon et al., 2009

I: Irritancy (contact excitation), R: Repellency (noncontact/spatial), R: Resistant, S: Susceptible to test compound
++++: 81-100% escaped from treated chamber
+++: 61-79% escaped from treated chamber
++: 41-59% escaped from treated chamber
+: 21-40% escaped from treated chamber
-: $\leq$ 20% escaped from treated chamber but statistically significant ($P < 0.05$) from the matched control

Table 2. Degree of behavioral responses of female *Aedes aegypti* to synthetic pyrethroids and DDT at an operational field dose (mg/cm²).

detect a chemical on direct contact or spatially before acquiring a lethal dose (Muirhead-Thomson, 1960). On the other hand, a stimulus-independent response does not require direct sensory stimulation of insect for avoidance to occur but rather involves other natural behavioral components such as exophily (outside resting) or zoophily (non-human blood preference) in which an insect avoids exposure to a chemical by preferentially utilizing habitats without active ingredients present (Byford & Sparks, 1987). This type of response has also been included in so-called "phenotypic and genotypic behaviors" (WHO, 1986). Stimulus-dependent behavioral responses include the avoidance behaviors discussed in detail in this chapter. The term *avoidance behavior* is generally used to describe actions that are stimulated by some combination of excitation (irritancy) and repellency, the former taking place following physical contact while spatial repellency results without physical contact with an insecticide (Roberts et al., 1987).

Populations	Deltamethrin		Fenitrothion		Propoxur		References
	I	*R*	*I*	*R*	*I*	*R*	
Bangkok*	++	-	++	-	+	-	Sathantriphop et al., 2006
Nontaburi*	+	-	+	-	+	-	Sathantriphop et al., 2006
Mae Sot*	+	-	+	-	+	-	Sathantriphop et al., 2006

*Highly resistance to deltamethrin
I: Irritancy, R: Repellency
++++: 81-100% escaped from treated chamber
+++: 61-79% escaped from treated chamber
++: 41-59% escaped from treated chamber
+: 21-40% escaped from treated chamber
-: $\leq$ 20% escaped from treated chamber but statistically significant ($P < 0.05$) from the matched control

Table 3. Degree of behavioral responses of *Culex quinquefasciatus* to deltamethrin, propoxur and fenitrothion at an operational field dose (mg/cm^2).

4. Conclusion

Any compound used to control (eliminate, reduce or otherwise prevent harm) insect populations have been termed an "insecticide". An insecticide can also be defined as any compound that is used solely to kill insects. This single term of reference involving only one type of action (knockdown/death) is completely inadequate to describe the more meaningful and complete action of many insecticide compounds used against insect populations. Insects can respond to insecticides in at least 2 different ways; behavioral action, namely avoidance and toxicity. In the past, the prevailing practice has been to classify chemicals simply as toxicants for killing insects. As seen in this chapter, we introduce the term "chemical" in place of "insecticide" as it is more appropriate for recognizing the 2 other primary actions on mosquitoes vice toxicity alone.

Chemicals protect humans from the bites of mosquitoes through 3 different actions: excitation, repellency and toxcity (Roberts et al., 2000 & Grieco et al., 2007). Historically the vast majority of chemical studies have focused on the direct toxicological responses (susceptibility and resistance) of chemicals on mosquito populations whereas very little emphasis has been placed on the vector's behavior in response to sub-lethal exposure. Knowledge of the mosquito's behavioral responses to particular chemicals is highly significant in the prioritization and design of appropriate vector prevention and control

strategies. Today, the development of insecticide resistance in insect pests and disease vectors occurs worldwide and on a increasing scale. However, resistance has still remained limited in many areas in spite of the long-term use of chemicals for control. This phenomenon suggests that behavioral responses likely play a significant role in how certain chemcials preform to interrupt human-vector contact while also reducing the selection pressure on a target insects for developing resistance (Roberts et al., 2000).

As discussed, at least 2 different types of mosqutio behavioral response outcomes to chemicals are recognized; excitation and repellency. Whether acting from direct contact or from a distance (spatially) both response activities are based on stimulus-response actions that result in clear movement away from an area with the chemical present. There have been numerous attempts to accurately measure the behavioral responses of mosquitoes to chemicals using various types of excito-repellency test systems (e.g., ERB and HITSS). However, no single system has yet been completely accepted as a standardized method of testing or analyzing behavioral responses. Currently, no test system as recommended by the WHO can discriminate between contact irritancy and noncontact repellency. The WHO tests are based on the concept that mosquitoes respond to chemicals only after physical contact and that spatial repellency plays little or no meaningful role in disease control, or may actually be a determental attribute of a chemical. The test systems describe in this chapter have the capacity to differentiate both key behavioral responses and thus assigning each with relative importance in the potential prevention of disease transmission. Both the ERB and HITSS systems have been used to study behavioral responses of several important mosquito vectors and pests (*Anopheles, Aedes,* and *Culex*) to various test chemicals currently used in vector control programs while providing valuable and highly reproducible results. This knowledge will allow better decision making on chemical selection, application method and future product development. From the detailed investigations previously mentioned and elsewhere, we conclude that the behavioral responses of mosquitoes to chemicals are an important, if not critical, components of disease control operations.

5. Acknowledgments

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6. References

Bangs, M.J. (1999). The Susceptibility and Behavior of *Anopheles albimanus* Weidemann and *Anopheles vestitipennis* Dyar & Knab (Diptera: Culicidae) to Insecticides in Northern Belize, Central America. Doctoral Dissertation, Uniformed Services University of the Health Sciences, Bethesda, Maryland, pp. 448.

Bondareva, N.L.; Artem'ev, M.M. & Gracheva, G.V. (1986). Susceptibility and Irritability Caused by Insecticides to Malaria Mosquitoes in the USSR. Part 1. *Anopheles pulcherrimus*. *Meditsinskaia Parazitologha I Parazitarnye Bolezni* (Moskva),Vol. 6, pp. 52-55.

Brown, A.W.A. (1983). Insecticide Resistance as a Factor in the Integrated Control of Culicidae. In Integrated Mosquito Control Methodologies. Volume 1. Laird M & Mile J. Eds. Academic Press: New York, pp. 161-235.

Byford, R.L & Sparks, T.C. (1987). Chemical Approaches to the Management of Resistant Horn Fly, *Haematobia irritans* (L.) Populations. *In* M. 0. Ford, D. W. Holloman, B. P. S. Khambay and R. M. Sawicki (eds.), Combating Resistance to Xenobiotics: Biological and Chemical Approaches, pp. 178-189. Ellis Horwood: Chichester UK.

Chareonviriyaphap, T.; Roberts, D.R., Andre, R. G., Harlan, H. & Bangs, M.J. (1997). Pesticide Avoidance Behavior in *Anopheles albimanus* Wiedemann. *Journal of the American Mosquito Control Association,* Vol. 13, No. 2, pp. 171-183, ISSN 1046-3607.

Chareonviriyaphap, T. & Aum-Aung, B. (2000). An Improved Excito-Repellency Escape Chamber for Behavioral Test in Mosquito Vectors. *Mekong Malaria Forum*, Vol. 5, pp.82-87.

Chareonviriyaphap, T.; Sungvornyothin, S., Ratanatham, S., & Prabaripai, A. (2001). Pesticide-Induced Behavioral Responses of *Anopheles minimus*, a Malaria Vector in Thailand. *Journal of the American Mosquito Control Association*, Vol. 17, No.1, (March 2001), pp, 13-22, ISSN 1046-3607.

Chareonviriyaphap, T.; Prabaripai, A., & Sungvornyothin, S. (2002). An Improved Excito-Repellency for Mosquito Behavioral Test. *Journal of Vector Ecology*, Vol. 27, No.2, (December 2002), pp, 250-252, ISSN 1081-1710.

Chareonviriyaphap, T.; Prabaripai, A. & Bangs, M.J. (2004). Excito-Repellency of Deltamethrin on the Malaria Vectors, *Anopheles minimus, Anopheles dirus, Anopheles sawadwongporni,* and *Anopheles maculatus*, in Thailand. *Journal of the American Mosquito Control Association,* Vol. 20, No.1, (March 2004), pp. 45-54, ISSN 1046-3607.

Chareonviriyaphap, T.; Parbaripai, A., Bangs, M.J., Kongmee, M., Sathantriphop, S., Muenvorn, V., Suwonkerd, W. & Akratanakul, P. (2006). Influence of Nutritional and Physiological Status on Behavioral Responses of *Aedes aegypti* (Diptera: Culicidae) to Deltamethrin and Cypermethrin. *Journal of Vector Ecology*, Vol. 31, No.1, (June 2006), pp. 89-101, ISSN 1081-1710.

Charlwood, J.D. & Paraluppi, N.D. (1978). The Use of Excito-Repellency Box with *Anopheles darlingi* Root, *An. nuneztovari* Gabaldon and *Culex pipiens quinquefaciatus* Say, Obtained from the Areas near Manaus, Amazonas. *Acta Amazonica*, Vol. 8, No. 4, pp. 605-611, ISSN 0044-5967.

de Zulueta, J. & Cullen, J.R. (1963). Deterrent Effect of Insecticides on Malaria Vectors. *Nature,* Vol.200, (November 1963), pp. 860-61, ISSN 0028-0836.

Evans, R. G. (1993). Laboratory Evaluation of the Irritancy of Bendiocarb, Lambdacyhalothrin, and DDT to *Anopheles gambiae. Journal of the American Mosquito Control Association,* Vol. 9, No.3, (September 1993), pp. 285-293, ISSN 1046-3607.

Elliott, R. & de Zulueta, J. (1975). Ethological Resistance in Malaria Vectors. Behavioral Response to Intradomestic residual insecticides. WHO/VBC75.569, pp. 1-15.

Gahan, J.B. & Lindquist, A.W. (1945). DDT Residual Sprays Applied in Buildings to Control *Anopheles quadrimaculatus*. *Journal of Economic Entomology,* Vol.38, No.2, pp. 223-230, ISSN 0022-0493.

Georghiou, G.P. (1972). The Evolution of Resistance to Pesticides. *Annual Review of Ecology and Systematics*, Vol.3, No.1 (November 1972), pp. 133-168, ISSN 0066-4162.

Giglioli, G. (1948). An Investigation of the House-Frequenting Habits of Mosquitoes of the British Guiana Coastland in Relation to the Use of DDT. *American Journal of Tropical Medicine and Hygiene,* Vol. 28, No.1, (January 1948), pp. 43-70, ISSN 0002-9637.

Grieco, J.P.; Achee, N.L., Andre, R.G. & Roberts, D.R. (2000). A Comparison Study of House Entering and Exiting Behavior of *Anopheles vestitipennis* (Diptera: Culicidae) using Experimental Huts Sprayed with DDT or Deltamethrin in the Southern District of Toledo, Belize, CA. *Journal of Vector Ecology,* Vol. 25 No.1, (June 2000), pp. 62-73, ISSN 1081-1710.

Grieco, J.P.; Achee, N.L., Sardelis, M.R., Chauhan, K .R. & Roberts, D.R. (2005). A Novel High Throughput Screening System to Evaluate the Behavioral Response of Adult Mosquitoes to Chemicals. *Journal of the American Mosquito Control Association*, Vol. 21, No.4, (December 2005), pp. 404-411, ISSN 1046-3607.

Grieco, J.P.; Achee, N.L., Chareonviriyaphap, T., Suwonkerd, W., Chauhan, K.R., Sardelis, M. & Roberts, D.R. (2007). A New Classification System for the Actions of IRS Chemicals Traditionally Used for Malaria Control. *PLoS ONE*, Vol.2, Issue. 8, (August 2008), pp. e716, ISSN 1932-6203.

Gubler, D.J. (1997). Dengue and Dengue Haemorrhagic Fever: Its History and Resurgence as a Global Public Health Problem. In: Gubler, DJ and Kuno, G (eds.). Dengue and Dengue Haemorrhagic Fever. New York: CAB International. 22 pp.

Kennedy, J.S. (1947). The Excitant and Repellent Effects on Mosquitoes of Sub-lethal Contacts with DDT. *Bulletin of Entomological Research,* Vol. 37, No. 4, (March 1947), pp. 593-607, ISSN 0007-4853.

Kongmee, M.; Prabaripai, A., Akaratanakul, P., Bangs, M.J. & Chareonviriyaphap, T. (2004). Behavioral Responses of *Aedes aegypti* (Diptera: Culicidae) Exposed to Deltamethrin and Possible Implications for Disease Control. *Journal of Medical Entomology*, Vol. 41, No. 6, (November 2004) pp.1055-1063, ISSN 0022-2585.

Lindsay, S.W.; Adiamah, J.H., Miller, J.E. & Armstrong, J.R.M. (1991). Pyrethroid-Treated Bednet Effects on Mosquitoes of *Anopheles gambiae* Complex in the Gambia. *Medical and Veterinary Entomology,* Vol. 5, No.4, (October 1991), pp. 477-483, ISSN 0269-283X.

Lockwood, J.A.; Sparks, T.C. & Story, R.N. (1984). Evolution of Insect Resistance to Insecticide: a Reevaluation of the Roles of Physiology and Behavior. *Bulletin of the Entomological Society of America,* Vol. 30, No. 4, (November 1984), pp. 41-51, ISSN 0013-8754.

Malaithong, N.; Polsomboon, S., Poolprasert, P., Parbaripai, A., Bangs, M.J., Suwonkerd, W., Pothikasikorn, J., Akratanakul, P. & Chareonviriyaphap, T. (2010). Human-Landing Patterns of *Anopheles dirus* sensu lato (Diptera: Culicidae) in Experimental Huts Treated with DDT or Deltamethrin. *Journal of Medical Entomology,* Vol. 47, No. 4, (September 2010), pp. 823-32, ISSN 0022-2585.

Metcalf, R.L.; Hess, A.D., Smith, G.E., Jeffery, G.M. & Ludwig, G.W. 1945. Observations on the Use of DDT for the Control of *Anopheles quadrimaculatus*. Public Health Report, Vol. 60, No.27, pp. 753-774, ISSN 0033-3549.

Miller, J.E. (1990). Laboratory and Field Studies of Insecticide Impregnated Fibers for Mosquito Control. Ph.D. Dissertation, University of London. 336 pp.

Miller, J. R.; Siegert, P.Y., Amimo, F.A. & Walker, E.D. (2009). Designation of Chemicals in Terms of the Locomotor Responses They Elicit from Insects: an Update of Dethier et al. (1960). *Journal of Economic Entomology,* Vol. 102, No. 6, (December 2009), pp. 2056-2060, ISSN 0022-0493.

Mongkalangoon, P.; Grieco, J.P., Achee, N.L., Suwonkerd, W. & Chareonviriyaphap, T. (2009). Irritability and Repellency of Synthetic Pyrethroids on an *Aedes aegypti* Population from Thailand. *Journal of Vector Ecology*, Vol. 34, No. 1, (June 2009), pp. 217-24, ISSN 1081-1710.

Muenworn, V.; Akaratanakul, P., Bangs, M.J., Parbaripai, A. & Chareonviriyaphap, T. (2006). Insecticide Induced Behavioral Responses in two Populations of *Anopheles maculatus* and *Anopheles swadwongporni*, Malaria Vectors in Thailand. *Journal of the American Mosquito Control Association*, Vol. 22, No. 4, (December 2006), pp. 689-698, ISSN 1046-3607.

Muirhead-Thomson, R.C. (1951). Mosquito Behaviour in Relation to Malaria Transmission and Control in the Topics. pp. viii+219; 16 plates: 22 Text Figure. London: Arnold.

Muirhead-Thomson, R.C. (1960). The Significance of Irritability, Behaviouristic Avoidance and Allied Phenomena in Malaria Eradication. *Bulletin of World Health Organization*, Vol. 22, pp. 721-734, ISSN 0042-9686.

Peaporn, P.; Supaphathom, K., Sathantriphop, S., Chareonviriyaphap, T., Yaicharoen, R. (2007). Behavioral Responses of Deltamethrin and Permethrin Resistant Strains of *Aedes aegypti* When Exposed to Permethrin in an Excito-Repellency Test System. *Dengue Bulletin*, Vol. 31, pp. 153-159, ISSN 0250-8362.

Pates, H. & Curtis, C. (2005). Mosquito Behavior and Vector Control. *Annual Review of Entomology*, Vol. 50, pp.53–70, ISSN 0066-4170.

Polsomboon, S.; Poolprasert, P., Suwonkerd, W., Bangs, M.J., Tanasinchayakul, S., Akratanakul, P. & Chareonviriyaphap, T. (2008). Biting Patterns of *Anopheles minimus* Complex (Diptera: Culicidae) in Experimental Huts Treated with DDT and Deltamethrin. *Journal of Vector Ecology,* Vol. 33, No. 2, (December 2008), pp. 285-292, ISSN 1081-1710.

Pothikasikorn, J.; Chareonviriyaphap, T., Bangs, M.J. & Prabaripai, A. (2005). Behavioral Responses to DDT and Pyrethroids between *Anopheles minimus* Species A and C, Malaria Vectors in Thailand. *American Journal of Tropical Medicine and Hygiene*, Vol. 73, No. 2, (August 2005) pp. 343-349, ISSN 0002-9637.

Pothikasikorn, J.; Overgaard, H., Ketavan, C., Visetson, S., Bangs, M.J. & Chareonviriyaphap, T. (2007). Behavioral Responses of Malaria Vectors, *Anopheles minimus* Complex, to Three Classes of Agrochemicals in Thailand. *Journal of Medical Entomology,* Vol.44, No. 6, (November 2007), pp. 1032-1039, ISSN 0022-2585.

Quinones, M.L. & Suarez, M.F. (1989). Irritability to DDT of Natural Populations of the Primary Malaria Vectors in Colombia. *Journal of the American Mosquito Control Association*, Vol. 5, No. 1, (March 1989), pp. 56—59, ISSN 1046-3607.

Rachou, R.G. ; Moura- Lima, M., Duret, J.P.& Kerr, J.R. (1963). Experiences with the Excito-Repellency Test Box—Model OPS, pp. 442-447. *In* Proceedings, 50th Annual Meeting of the New Jersey Mosquito, the 19th American Mosquito Control Association, ISSN 1046-3607.

Ree, H.I. & Loong, K.P. (1989). Irritability of *Anopheles farauti, Anopheles maculatus*, and *Culex quinquefasciatus* to Permethrin. *Japanese Journal of Sanitary Zoology*, Vol. 40, No. 1, pp. 47—51, ISSN 0424-7086.

Reiter, P. & Gubler, D.J. (1997). Surveillance and Control of Urban Dengue Vectors. In: Gubler, DJ and Kuno, G (eds.). Dengue and Dengue Haemorrhagic Fever. New York: CAB International. pp. 425-462

Roberts, D.R. (1993). Insecticide Repellency in Malaria Vector Control: a Position Paper. VBC Report No. 81131. United States Agency for International Development, Arlington, VA. 72 pp, ISSN 0736-718X.

Roberts, D.R.; Alecrim, W.D., Tavares, A.M. & McNeil, K.M. (1984). Influence of Physiological Condition on the Behavioral Responses of *Anopheles darlingi* to DDT. *Mosquito News,* Vol.44, No.3, pp. 357-361, ISSN 0027-142X.

Roberts, D.R.; Alecrim, W.D., Tavares, A.M. & Radke, M.G. (1987). The House-Frequenting, Host Seeking and Resting Behavior of *Anopheles darlingi* in Southeastern Amazonas, Brazil. *Journal of the American Mosquito Control Association,* Vol.3, No.3, (September 1997), pp. 433-441, ISSN 1046-3607.

Roberts, D.R. & Alecrim, W.D. (1991). Behavioral response of *Anopheles darlingi* to DDT sprayed house walls in Amazonia. *The Bulletin of the Pan American Health Organization*, Vol.25, No. 3, pp. 210—217, ISSN 0042-9686.

Roberts DR, & Andre RG. (1994). Insecticide Resistance Issues in Vector Borne Disease Control. *American Journal of Tropical Medicine and Hygiene*, Vol.50, No.6, pp. 21—34, ISSN 0002-9637.

Roberts, D.R.; Chareonviriyaphap, T., Harlan, H.H. & Hshieh, P. (1997). Methods for Testing and Analyzing Excito-Repellency Responses of Malaria Vectors to Insecticides. *Journal of the American Mosquito Control Association*, Vol.13, No.1, (March 1997), pp. 13-17, ISSN 1046-3607.

Roberts, D.R.; Alecrim, W.D., Hshieh, P., Grieco, J., Bangs, M.J., Andre, R.G. & Chareonviriyaphap, T. (2000). A Probability Model of Vector Behavior: Effects of DDT Repellency, Irritability, and Toxicity in Malaria Control. *Journal of Vector Ecology,* Vol.25, No. 1, (June 200), pp.48-61, ISSN 1081-1710.

Roberts, D.R. & Tren, R. (2010). The Excellent Powder: DDT's Political and Scientific History. Dog Ear Publishing, pp. 432, ISBN: 978-160844-376-5.

Rozendaal, J.A.; Van Hoof, J.P.M., Voorham, J. & Oostburg, B.F.J. (1989). Behavioral Responses of *Anopheles darlingi* in Suriname to DDT Residues on Housewalls. *Journal of the American Mosquito Control Association*, Vol.5, No. 3, (September 1989), pp. 339-350, ISSN 1046-3607.

Sathantriphop, S.; Ketavan, C., Prabripai, A., Vietson, S., Bangs, M.J., Akatanakul, P. & Chareonviriyaphap, T. (2006). Susceptibility and Avoidance Behavior by *Culex quinquefasciatus* Say to three Classes of Residual Insecticides. *Journal of Vector Ecology,* Vol. 31, No. 2, (December 2006), pp. 266-274, ISSN 1081-1710.

Service, M.W. & Townson, H. (2002). The *Anopheles* Vector. pp. 59-84. In: Warrell DA, Gilles HM (eds). Essential malariology (4th ed.). Arnold, London UK, ISBN 9780340740644.

Smith, A. (1965). A Verandah-Trap Hut for Studying the House Frequenting Habits of Mosquitoes and for Assessing Insecticides. II. The Effect of Dichlorvos (DDVP) on Egress and Mortality of *Anopheles gambiae* Giles and *Mansonia uniformis* (Theo.) entering naturally. *Bulletin of Entomological Research*, Vol. 56, pp. 275-286, ISSN 0007-4853.

Smith, A. & Webley, D.J. (1969). A Varandah-Type Hut for Studying the House-Frequenting Habits of Mosquitoes and for Assessing Insecticide. III. The Effect of DDT on Behaviour and Mortality. *Bulletin of Entomological Research*, Vol. 59, No. 3, pp. 33-46, ISSN 0007-4853.

Sinka, M.E.; Bangs, M.J., Manguin, S., Chareonviriyaphap, T., Patil, A.P., Temperley, W.H., Gething, P.W., Elyazar, I.R., Kabaria, C.W., Harbach, R.E. & Hay, S.I. (2011). The Dominant *Anopheles* Vectors of Human Malaria in the Asia-Pacific Region: Occurrence Data, Distribution Maps and Bionomic Précis. *Parasites Vectors* , Vol.4, (May 2011), pp. 89, ISSN 1756-3305.

Sungvornyothin, S.; Chareonviriyaphap, T., Prabaripai, A., Trirakhupt, T., Ratanatham, S. & Bangs, M.J. (2001). Effects of Nutritional and Physiological Status on Behavioral Avoidance of *Anopheles minimus* (Diptera: Culicidae) to DDT, Deltamethrin and Lambdacyhalothrin. *Journal of Vector Ecology*, Vol. 26, No.2, (December 2001), pp. 202-215, ISSN 1081-1710.

Suwannachote, N.; Grieco, J.P., Achee, N.L., Suwonkerd, W., Wongtong, S. & Chareonviriyaphap, T. (2009). Effects of Environmental Conditions on the Movement Patterns of *Aedes aegypti* (Diptera: Culicidae) into and out of Experimental Huts in Thailand. *Journal of Vector Ecology*, Vol. 34, No. 2, (December 2009), pp. 267-275, ISSN 1081-1710.

Tanasinchayakul, S.; Polsomboon, S., Prabaripai, A. & Chareonviriyaphap, T. (2006). An Automated, Field Compatible Device for Excito-Repellency Assay in Mosquitoes. *Journal of Vector Ecology*, Vol.31, No. 1, (June 2006), pp. 210-212, ISSN 1081-1710.

Thanispong, K.; Achee, N.L., Bangs, M.J., Grieco, J.P., Suwonkerd, W., Prabaripai, A., & Chareonviriyaphap, T. (2009). Irritancy and Repellency Behavioral Responses of three strains of *Aedes aegypti* Exposed to DDT and Alpha-cypermethrin. *Journal of Medical Entomology*, Vol. 46, No. 6, (November 2009), pp. 1407-1414, ISSN 0022-2585.

Thanispong, K.; Achee, N.L., Grieco, J.P., Bangs, M.J., Suwonkerd, W., Prabaripai, A., Chauhan, K.R. & Chareonviriyaphap, T. (2010). A High Throughput Screening System for Determining the three Actions of Insecticides against *Aedes aegypti* (Diptera: Culicidae) Populations in Thailand. *Journal of Medical Entomology,*Vol. 47, No. 5, (September 2010), 833-841, ISSN 0022-2585.

Tisgratog, R.; Thananchai, C., Bangs, M.J., Thainchum, K., Juntarajumnong, W., Prabaripai, A., Chauhan, K.R., Pothikasikorn, J.,& Chareonviriyaphap, T. (2011). Chemical Induced Behavioral Responses in *Anopheles minimus* and *Anopheles harrisoni* in Thailand. *Journal of Vector Ecology*, Vol. 36, No.2 (December 2011), pp. xxx-xxx, ISSN 1081-1710.

Trapido, H. (1954). Recent Experiments on Possible Resistance to DDT by *Anopheles albimanus* in Panama. *Bulletin of the World Health Organization*, 11: 885-889, ISSN 0042-9686.

World Health Organization. (1970). Insecticide Resistance and Vector Control. 17th Report of the World Health Organization Expert Committee on Insecticide. Instructions for Determining the Irritability of Adult Mosquitoes to Insecticide. *World Health Organization Technical Report Series,* pp. 433. ISSN 0512-3054.

World Health Organization. (1986). Resistance of Vectors and Reservoirs of Disease to Pesticides: *Tenth Report of the World Health Organization Offset Publication*, No 13, ISSN 0303-7878.

World Health Organization. (1992). Vector Resistance to Pesticides. Technical Report Series No. 818. *World Health Organization*, Geneva, pp. 62, ISSN 0042-9686.

World Health Organization. (1999). Prevention and Control and Dengue and Dengue Haemorrhagic Fever: Comprehensive Guidelines. *World Health Organization* Regional Publication, SEARO No. 29, New Delhi., pp. 134, ISSN 0378-2255.

10

Essential Plant Oils and Insecticidal Activity in *Culex quinquefasciatus*

Maureen Leyva, Olinka Tiomno, Juan E. Tacoronte,
Maria del Carmen Marquetti and Domingo Montada
Institute Tropical "Pedro Kouri"
Cuba

1. Introduction

Plants have great importance for man because they are one of their sources of food, they provide us through the process of photosynthesis the oxygen we breathe and are essential to maintain the ecological balance (Corbino, 2000).

Essential oils are volatile, usually distillable liquid fractions responsible for the aroma of the plant. The vast majority of them are pleasant smell and its metabolic and evolutionary significance lies in the role they play as attractor of pollinating agents (for its pleasant aroma), constitute elements of defense against the attack of parasites, herbivorous animals and insects, allow the adaptation of the plant when water is scarce and are part of the substances in reserve as the giver of H+ in the processes of electron. The organoleptic characteristics of the essential oils may be given by major components, although in other cases they are substances present in tiny quantities (traces) which define the taste, smell, or therapeutic properties (Scholes, 1995; Worwood, 1992).

These natural substances are known as secondary metabolites, name which refers to substances that are not involved in the basic mechanisms of life of the plant but that comply with specific functions. (Corbino, 2000). They tend to accumulate in large amounts without negative effects and represent a problem in the cell or on the plant. These metabolites have the property to form glycosides and are found soluble in the plant. For many years these metabolites were regarded as final products of metabolic processes without specific function or directly as a waste of plant products (Lopez, 2008).

The study of these substances was initiated by organic chemists of the 19th century and early 20th century who were interested in these substances because of its importance in the medical industry, the manufacture of flavoring, etc. In fact, the study of the secondary metabolites stimulated the development of separating techniques, and spectroscopy for determining their structure and synthesis which constituted the basis of the contemporary organic chemistry (Lopez, 2008).

They can be found in different parts of the plant: leaves (wormwood, basil, buchú, cidrón, eucalyptus, mint, lemongrass, marjoram, mint, patchouli, quenopod, rosemary, Sage, lemon balm, etc.), in the roots (angelic, asaro, saffron, calamus, turmeric, galanga, ginger, sandalwood, Sasafras, Valerian, vetiver, etc.), in the pericarp of the fruit (lemon, Tangerine, Orange, etc.), seeds (anise, cardamom, dill, fennel, cumin, etc.), in the stem (cinnamon, caparrapí, etc.), flowers (arnica, lavender, chamomile, pyrethrum, thyme, clove scent, rose,

etc.) and in the fruit (caraway, coriander, bay leaf, nutmeg, parsley, pepper, etc.). (www.buscasalud.com)

2. Essential oils

Essential oils are complex mixtures of up to over 100 secondary metabolites, which can be classified in terpenoid compounds, phenolic and alkaloids based on their biosynthetic origins. Terpenes containing a single unit of isoprene are called monoterpenes, which contain three units named sesquiterpenes (Ikan, 1991; Silva, 2002).

Monoterpenes and sesquiterpenes are terpenes from 10 to 15 atoms of carbons biosynthetically derived from geranilpyrophosfates (SPG) and farnesilpyrophosfate (FPP) respectively. Monoterpenes and in general all the natural terpenoid compounds are synthetized by the route of the acetilCoA through a common intermediate which is the mevalonic acid. However, it has been proposed that some terpenoids are not originated by this route, and instead use an alternative route that may involve pyruvate, glyceraldehyde-3-phosphate (Fig 1) (Adams et al, 1998; Sponsel, 1995).

Essential oils are widely distributed in some 60 families of plants including the Compositae, Labiadas, Lauraceae, Myrtaceae, Rosaceae, Rutaceae, Umbelliferae, etc. Monoterpenoides are mainly found in plants in the family of Ranunculales, Violales, and Primulales, while they are rare in Rutales, Cornales, Lamiales, and Asterales. Moreover, the sesquiterpenoides are commonly found in Rutales, Cornales, Magnoliales and Asterales. Although in both essential oils: mono - sesquiterpenes and phenylpropan are found in free-form, those which are linked to carbohydrates, have been more recently investigated, because it is considered to be the immediate precursor of the essential oil.

From the chemical point of view, despite its complex composition with different types of substances, essential oils can be classified according to the type of substances of which they are the major components. According to this fact, the essential oils rich in monoterpenes are called essential oils sesquiterpenoids, those rich in phenylpropan are the essential oils phenylpropanoids. Although this classification is very general there are more complex classifications that take into account other chemical aspects (Fig 2,3,4) (González, 1984; Ikan, 1991; Judd et al, 2002; Stachenko, 1996).

2.1 Methods of extraction

According to www.chemkeys.com 2000; Gascon et al. 2002; www.herbotecnia. com.ar, 2002; www.losandes.com.ar 2002. essential oils can be extracted from plant samples through various methods samples such as: expression, distillation with steam, extraction with volatile solvents, enfleurage, extraction with supercritical fluids and hydrodistillation.

2.2 Method of isolation

From such oils, it is possible to its isolation through the use of one or several chromatographic methods such as thin layer chromatography and HPLC.

For the chromatographic column and thin layer methods the silica gel are widely used as stationary phase. Polar pure or mixed solvents such as: Toluene-acetate of ethyl benzene, chloroform, dichloromethane, ethyl, chloroform-benzene, chloroform-ethanol - chloroform-benzene acetic acid benzene-acetate are used as mobile phase.

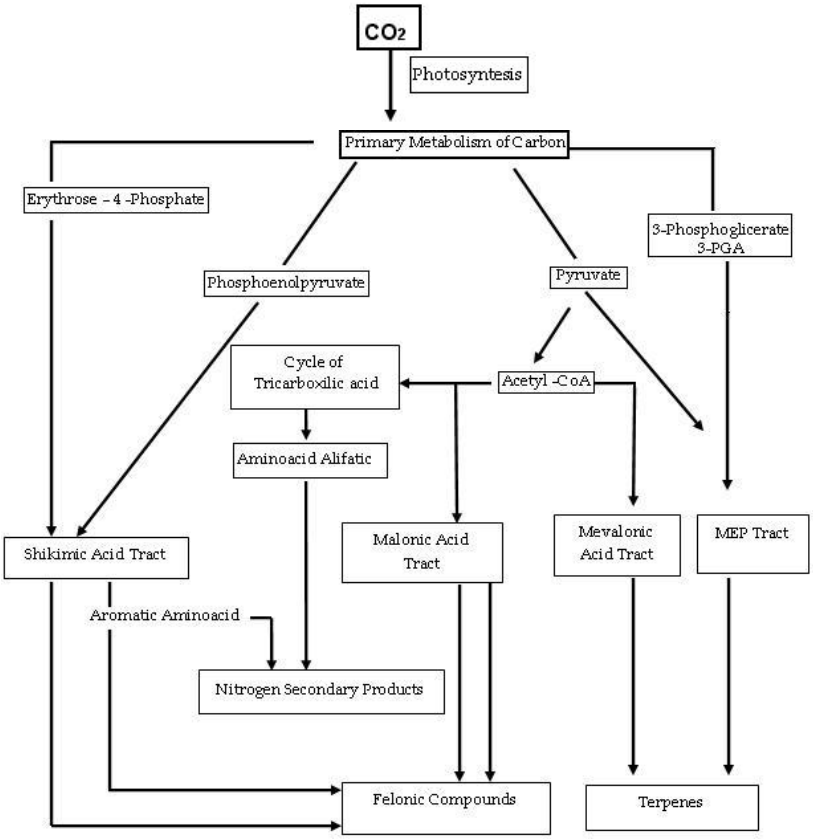

Fig. 1. General pathways of plant secondary metabolism.

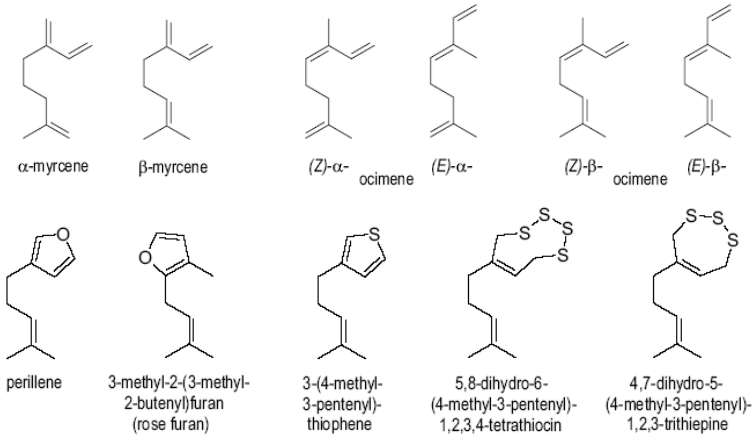

Fig. 2. Natural Monoterpenes.

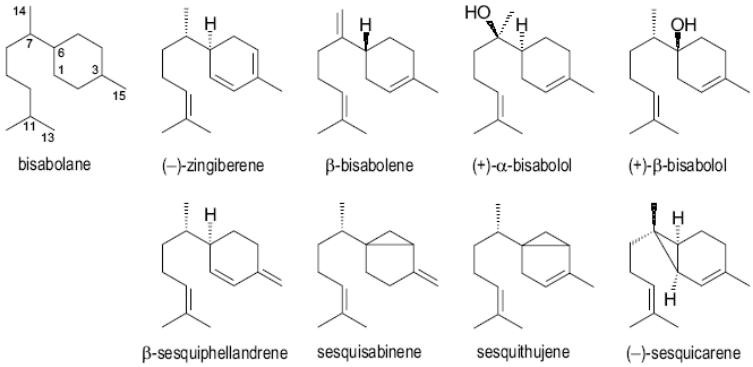

Fig. 3. Natural Sesquiterpenes.

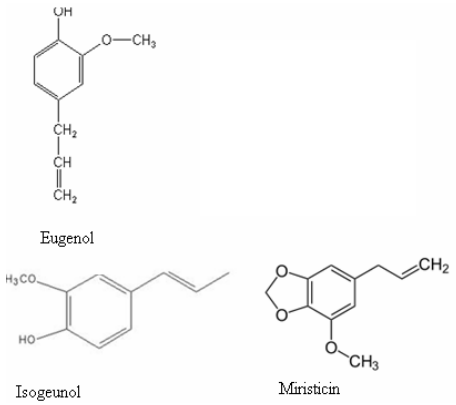

Fig. 4. Naturals phenylpropanoids.

However, currently more efficient and faster techniques of separation are used such as HPLC high efficiency liquid chromatography and gas (CG) chromatography, as well as also combinations "ON-LINE" HPLC- CG EM. These same methods are used for the analysis of the flower essences. The last technique, thanks to the recent development of capillary columns for high resolution, allows analyzing complex mixtures present in essential oils, and identify the components from the retention times through the so-called Kovats retention indexes (Ik). These values are characteristic for each component and there are databases with indexes of many components of essential oils. Ik values are determined in two chromatographic columns, one polar (e.g. CARBOWAX 20 M) and a non-polar (e.g. OV - 101 also called DB-1).

In addition, the docked technique chromatography of gases - mass spectrometry, (GC-MS) allows obtaining the spectrum of mass of each component which builds the molecular weight and structural information. Likewise there are databases with spectra of masses of many components, something similar goes for the index Kovats (determined in two columns of different polarity) and the spectra of mass are criteria for chemical allocation of many components of essential oils not just monoterpenes but also other types of substances characteristic of such oils. More recently the column chromatographic Chiral for separation

of optically active components have been developed, and have developed methods for combined analysis HPLC HPLC-NMR of mixtures of sesquiterpenes and mass spectrometry.

2.3 Test of recognition of monoterpenes and sesquiterpenes (Pino, 1999; Stam, 1970)

Due to the diversity of functional groups that can be present in the components of mono - and sesquiterpenics of an essential oil, there is a specific test for their recognition. However there are few experimental procedures that allow to recognize some of them by their coloration with different reagents, its absorption of 254 nm UV light and its Rf in thin layer chromatography. For example, limonene is recognized in the plates of TLC because it does not absorb UV 254 nm light, adds bromine, does not form a derivative 2, 4-dinitrophenylhydrazone and produces Brown with sulfuric acid.

There is a procedure which combines the TLC with micro reactions (oxidation, reduction, dehydration, hydrolysis, etc.) described in the classic book already today of Ikan. Other reagents useful for revealing monoterpenes and sesquiterpenes are anisaldehid-sulfuric acid, sulfuric acid -vanillin and phosphomolibdic acid.

2.3.1 Spectral characterization

Monoterpenes and sesquiterpenes, in a number, can be characterized chemically from the gas chromatography and mass spectra data as noted above, but when there are doubts of such characterization some spectral methods such as infrared ultraviolet and magnetic resonance spectrometry can be used.

2.3.1.1 Infrared spectroscopy

Infrared Spectroscopy allows detection of the presence of groups hydroxyl, Carbonyl, aromatic rings, bond double C = C cis and trans, etc. To determine the spectrum just put a drop of the component in a cell of NaCl. For example, in the infrared spectrum of the 3-p-menten-7 present in the oil of cumin, the intense band in 1725 cm^{-1} indicates a group not conjugated Carbonyl. The peak at 2710 cm^{-1} is assigned to a proton aldehydic C-H. The bond around on 1375 cm^{-1} indicates a group isopropyl, and the band of 817 cm^{-1} average intensity indicates a double bond tri-substituted.

2.3.1.2 Ultraviolet spectroscopy

UV spectrum ultraviolet of monoterpenes and sesquiterpenes allows the recognition of functional groups and chromophoric. E.g. limonene presents an absorption maximum at 262 nm (ξ = 6400).

Nuclear magnetic resonance (^{1}H - 13 C); due to the developments of the NMR has database of Spectra, especially of 13C -NMR for the monoterpenes and more distributed sesquiterpenes. The NMR spectrometry-13 C has the additional advantage that the carbons of terpenoid chemical displacement (and other natural and synthetic substances) can be calculated by computer programs available in the market as Chemwind and ACD-Lab.

In addition, the recent development of two-dimensional methods homo - and heteronuclear, has allowed the fine structural determination of terpenoids and other natural substances, eliminating the ambiguity in the allocation of the observed signals.

2.3.1.3 Mass Spectrometry

There are databases that contain information and spectra of masses of the components of mono - and sesquiterpenoids of essential oils. These databases are now available in many

commercial instruments of analysis as a chromatograph of gases coupled with mass spectrometers.

2.4 Phenylpropan

Phenylpropanes are secondary metabolites derived from phenylalanine, which is first converted to cinnamic acid and after a series of hidroxilations lead to acid cumaric, caffeic acid, pherulic acid etc. The conversion of these acids to their corresponding esters produces some of the volatile components responsible for the fragrance of herbs and flowers (Lopez, 2008).

Phenylpropanes of essential oils are extracted with the same methodology described above for mono - and sesquiterpenes. However, due to its aromatic ring they have advantages in detection by TLC and HPLC because absorb ultraviolet light (254 nm) and do not require to be disclosed with chemical agents, or need to be derivative, and therefore can be isolated and analyzed more easily.

2.4.1 Recognition of phenylpropan

There are test assay for recognition to the aromatic ring as a reaction with formaldehyde and sulfuric acid. Likewise in the case of phenylpropan with phenolic hydroxyl these can be recognized by the test assay of ferric chloride, which produces green and blue coloring with phenolic substances in general.

Substances with aromatic ring, their infrared spectra show signs characteristic of these compounds and give information about the type of replacement of the aromatic ring in addition to the functional groups present in the molecule. For example, the spectrum of eugenol shows, among other, bands in 3500 (wide) due to the hydroxyl group, 1510 characteristic of aromatic, and three bands in 990, 920 and 938 cm^{-1} characteristics of a vinyl mono substituted group. The IR from the cinamaldehyde spectrum displays bands in (weak) 3330, 3050, 2820, 2750, 1660 (intense, due to the carbonyl group), 975, 740 and 695 cm^{-1} among others.

Unlike the majority of mono - and sesquiterpenes, the phenylpropanes absorb UV light with a maximum around 254 nm depending on the groups present in the molecule chromophors. For example, the isoeugenol shows maxima at 260 (15850) and 305 (7000), safrole in 286 nm, the myristicine at 276 nm, the isosafrole in 264 nm, trans- cinnamic acid in 273 nm and cis-cinnamic acid in 264 nm.

Phenylpropan ^{1}H-NMR spectra show signs of aromatic protons around 6-8 ppm whose multiplicities and coupling constants allow a clear structural assignment even with low resolution spectra. Trans-anethole there is a double signal around 1.9 ppm due to the protons from the methyl group, a singlet in 3.9 due to the protons of the methoxy group, a complex signal around 6.1 ppm due to the two protons olefinics provision trans each other, and a double around 6.9 ppm characteristic of 4 protons of an aromatic ring p-based.

2.5 Natural insecticides way-of-action (Silva 2002)

As growth regulators, this effect can manifest itself in several ways. The first is related to the molecules that inhibit the metamorphosis. Other compounds make the insect to have an earlier metamorphosis, occurring at a time which is not favorable. It has been observed that certain molecules can alter the function of the hormones that regulate these mechanisms so that there are insects with malformations, dead or sterile (Gunderson, 1985; Sangykurn, 1999).

Studies carried out from different concentrations of extract of Meliaceas show that this extract inhibits the feeding and negatively affects the development and survival of different species of insects. The anti-alimentary activity of this compound shows that at doses ranging from 5.5 to 27. 6 μg/cm² caused an inhibitory activity of more than 75% the insects. The way of action of these compounds extracted from various species of Meliaceas are taken from a combination between an anti feeding effect and post-digestive toxicity. (www.cannabiscultura.com)

The use of plants as repellents is an ancient knowledge. This practice is basically done with compounds that have odor or irritant effects such as chili and garlic. There are homemade recipes that describe the use of fennel (*Foniculum vulgare*), rude (*Ruta graveolens*) and eucalyptus (*Eucalyptus globules*) among other aromatic plants to repel moths of clothing.

2.5.1 Mode of action of the natural insecticide

Treatments with natural compounds such as essential oils or pure compounds (Awde & Ryan, 1992; Keane & Ryan, 1999;Ryan & Byrne, 1988) may cause symptoms that indicate neurotoxic activity including hyperactivity, seizures and tremors followed by paralysis (knock down), which are very similar to those produced by the insecticides pyrethroids (Kostyukovsky et al., 2002).

Acetyl cholinesterase enzyme catalyzes the hydrolysis of the neurotransmitter excess acetylcholine in the synaptic space between choline and acetic acid. It has been recognized that essential oils could their effect through ACE inhibition.

Many hydrophobic compounds incite the deactivation of protein and enzyme inhibition; the acetilcolinesterase (AchE) is an enzyme that is particularly susceptible to the hydrophobic inhibition (Ryan et al. 1992).

Is described in this context essential oil interfering with AchE are acting as potent of the central nervous system where all synapses cholinergic are virtually located (Bloomquist, 1999) of the cholinesterase inhibitors are known as anticolinesterasic, the chemical products that interfere with the action of this enzyme are potent neurotoxins (López, 2008).

The majority of plant insecticide are extracts made by a group of active compounds of diverse chemical nature, which is hardly found in the same concentrations so the pressure of selection on the plague will not be always the same, i.e. in general, insects take more to develop resistance to a blend of compounds than any of its components separately (Silva, 2003).

2.6 Future prospects

With the beginning of the new millennium, assessment of a large number of essential oils from native or typical flora has increased in many countries of the Americas and Africa due to its potential use as an alternative method of control.

There have been studies with oils in laboratory conditions reporting larvicide action in species of the genus *Culex, Anopheles*, *Aedes* (Ansari, 2005; Assarn, 2003; Albuquerque et al, 2004; Amer et al, 2006a ; Bassole et al., 2003; Carvalho et to 2003; Cavalcanti et al, 2004; Cetin et al, 2006; Cheng et to 2004; Chung et 2010 b Dharmagda et al, 2005; of Mendonca et al., 2005; Faley et al., 2005; Hafeez et 2011; Khandagle et al, 2001; Mathew & Thoppil 2011;Morais et al, 2006; Prajapati et al., 2005; Pushpanathan et al, 2006; Phasomkusolsil & Soowera 2010; Pitarokili et al, 2011; Raví et al, 2006; Tare et al, 2004; Tomas et al, 2004).

In the same way it has been evaluated the repellent capacity of some oils in the same species of mosquitoes; Amer et al, 2006b;Barnard et al, 2004; Byeoung-Soo, 2005; Caballero-

Gallardo, et-2011; Chang et al, 2006; Jaenson et al, 2006; Das et al., 2003; Kim et al, 2004; Oyedale et al, 2002; Odalo et al., 2005; Pohilt et 2011; Phasomkusolsil & Soowera 2010; Tawatsin et al, 2006; Trongtokit et al. 2005a; 2005b; Yang et al., 2005; Zhu et al, 2006;
Less has been evaluated adulticide effect found only two reports described by Chaiyasit et al 2006 and Miot et al 2004.

3. Studies developing in Cuba with essential oils for controlling of public health pests

In Cuba there is a large plant biodiversity with a lot of endemic species. Myrtaceas, Piperaceas, Zingiberaceae, Pinaceae families possess insecticidal action potential based to their essential oils. To this day, there have been verified in public health pests such as; *Blatella germanica*, *Musca domestica* and *Aedes aegypti* (Aguilera et al 2003; 2004;Leyva et al, 2007 a, b, 2008a, b; 2009a, b; 2010) and more recently in *Culex quinquefasciatus.*
Evaluated essential oils are essentially from *Pimenta racemosa, Eugenia melanadenia, Psidium rotundatum* and *Melaleuca leucadendron* of the botanical family Myrtaceae, *Piper auritum, Piper aduncum* of the family Piperacea, *Curcuma longa* in the family Zingiberaceae, *Artemisia abrotamum* in the family Asteraceae, *Pinus tropicalis* and *Pinus caribbaea* of the Pinacea family. A derivative of essential oil of pinaceas has also been evaluated; turpentine oil obtained by hidrodestylation of the resin of endemic pine trees, mostly composed of terpenes (α-and β-pinene and a modified turpentine where a part of the original turpentine oil was modified by photoizomerization of the α and β-pinene to compound of the type verbenone and pulegone.)
In all tested oils, there has been found necessary dosages that elicited from 5% to 95% of mortality. After testing CHI2 they have a probability greater than 0.05 by which can raise all the mortalities occurring in each are associated with the used dose.
Studies with these oils in larvae of *Aedes aegypti* show high insecticidal action by the low lethal concentrations obtained and the high slopes of the regression lines; however, in *Blatella germanica* and *Musca domestica* the bioassays were conducted in adults, showing a very low activity given the high lethal concentrations obtained.
Natural essences of plants owe its insecticide and repellent action to the presence in its composition of derivative monoterpenic as d-limonene, α -terpineol, β - myrceno, linolool, 1.8-cineole, 4-terpineol, thymol, carvacrol, α-pinene and β-pinene.) Studied oils possess structures lactones as the 1.8 cineole, 4-terpinol, α and β - pinene, cineole, safrole, turmenon, chavicol, eugenol, beta-phellandrene, longifoleno among others, which account for a 18-68% of its total membership, and are that they can be attributed the high insecticidal action in *Aedes aegypti*.
One aspect assessed for reasons of eco-sustainability was ovicidal and inhibitory action of the development by the oil of turpentine in larvae of *Ae aegypti*
The largest ovicidal effect was shown with the dose diagnosis of oil of turpentine with photochemical treatment. In a test of hypothesis of different proportions for each dose of oil, the diagnostic doses were compared with every CL_{95} finding differences between the doses tested with modified oil of turpentine for p=0.05 while for oil not modified there was only significant difference comparing the CL_{95} and the diagnostic doses. (p=0.05). By comparing the CL_{95} and the diagnostic dose, he found significant difference for a p = 0.05. If we compare both types of essences we may conclude that although modified turpentine oil presents a CL_{95} dose higher than oil without changing this it has a greater effect ovicidal and therefore more protector.

The percentage of hatching inhibition was 36.47% of the total number of larvae. The larvae completing their development to adults corresponded to 60.54% of surviving larvae. As a figure of interest the greatest number of semi emergency in adults occurred at 72 hours after exposure to the oil of turpentine (with a predominance of males). At 96 hour, the largest proportion was in favor of females, who became non-existent for this sex at 120 and 168 hours (5-7 d)

More recently, oil of turpentine and the bicyclic α-pinene obtained by fractional distillation of oil of turpentine have been evaluated in *Culex quinquefasciatus*, important vector of West Nile virus and encephalitis

The purification of the α-pinene was made using column chromatography in system of variable stationary phase (SiO_2, Al_2O_3, and their mixtures in dependence of the polarities of the mobile phases for elution and separation).) Molecular characterization was developed, using techniques of FTIR and NMR, as well as methodologies for its extension to field conditions type TLC (chromatography on thin layer in the presence of plates of glass with silica gel 200-G254 activated with AgNO3 / acetonitril via wet impregnation with methanol). The plates are eluted with mixtures of solvents of media polarity (ethyl-acetone - alcohol isopropyl acetate). Figure 5 shows the spectroscopic characterization of the α pinene. In table 1 and 2 is shown the concentrations finally used, the obtained mortalities and lethal concentrations that causes 50% to 95% percent of mortality, the slope of the regression line and diagnostic dose.

The lowest lethal concentrations were obtained with oils of turpentine and turpentine modified followed by *Curcuma longa, Psidium rotundatum* and *Chenopodium ambrosoide.* By applying a χ2-square test ($p < 0.05$) to the results of the bioassays, the results showed that the mortalities are associated with the used dose. The higher slopes of the lines were equal with the oil of turpentine, *Eugenia melanadenia, Psidium rotundatum* and *Chenopodium ambrosiode*, corresponding to the lower value of the slope to the terpene α-pinene. To the slope values obtained were applied a χ2 test finding significant difference between them for $p < 0.0001$, indicating that these oils have a different response for the same strain.

In our assessed results we find the insecticidal action of oil in *Cx quinquefasciatus* and is important to note that lethal concentrations obtained are higher than concentrations found in *Aedes aegypti* to these same oils (Leyva,2008a; 2009a,b). This response could be that this species has developed physiological resistance and some tolerance to insecticides with which it has active defense before any insecticide action.

Oil of *Curcuma longa* presents in its composition more than 50% of sesquiterpenes, apart from the monoterpenes and α -pinene to which can be attributed the insecticide action. If we compare studies conducted with *Curcuma aromatica* (Choochate et al., 2005) and *Curcuma zedoaria* in *Aedes aegypti* these oils show lower values of LC_{50} and CL_{99} (Champakaew et al, 2007).

Within the family Piperaceae, notably members of the genus Piper have noted insecticidal action, molluscicide (Chansang et al., 2005; Parmar et 1997) this is due to the presence of the secondary metabolites as alkaloids, phenylpropanoids, lignans, neolignanos, terpenes, flavononas, among others (Pino et al, 2004; Smith & Kassim 1979). *Piper aduncum* specifically shows in its composition a high percent of dilapiol, α-pinene and 1.8- cineole (Bottia et 2007). In Mexico studies were not mortalities obtained with a kind of Piper in *Culex quinquefasciatus* in aqueous extracts to 5 and 15% where used in the aerial part of plants (Perez-Pacheco et al 2004).

The larvicidal activity of *Chenopodium ambrosioide* was evaluated in *Ae aegypti*, with extracts metanolics to different concentrations resulting most important inhibition of pupal development and an increase in the time of larval development (Suparvarn et al 1974) has also been evaluated in mosquito repellent activity (Gillij et al 2008). *Ch ambrosiodes* has as one of its components majority the carvacrol reported in the literature as insecticide terpene (Silva et al 2008; Kordali et al 2008). *Pimenta racemosa, Eugenia melanadenia* and *Psidium rotundatum* presented in its composition more than 50% of monoterpenes and sesquiterpenes standing out in his majority composition 1,8 cineole, 4-terpineole and α-pinene terpenes with high insecticidal action (Pino et al, 2004,2005).

Oil of turpentine in *Aedes aegypti* obtained strong insecticide and inhibitory action of development in previous studies (Lucia et al, 2007; Leyva et al, 2009b 2010). In this study isolated terpene α -pinene (one of the major components of turpentine) was higher than the own turpentine lethal concentrations, this may be due to the synergistic effect with several monoterpens (α and β -pinene) at the same time and not isolated and evaluated by themselves in insects.

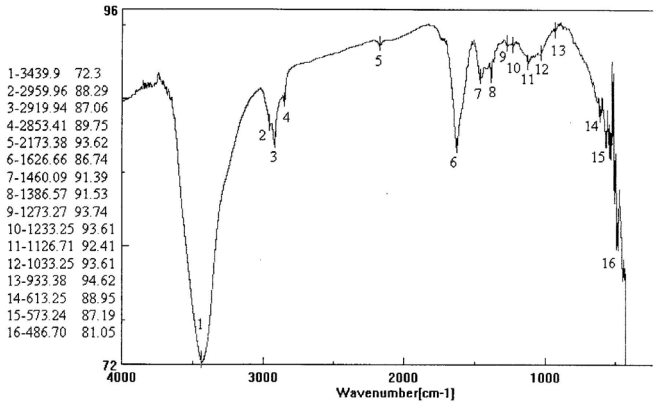

Fig. 5. Spectroscopic characterization of the α pinene.

Conc (%)	Mortality (%)	Lethal Conc		Slope of the Line
Turpentine				
0.0035	5	CL_{50}	0.0042	20.9
0.0040	31			Diagnostic
0.0050	94	CL_{95}	0.0055	Dose
0.0060	100			0.0108
				(108 mg/L)
Modified Turpentine				
0.0030	5	CL_{50}	0.0038	14.4
0.0040	60			Diagnostic
0.0050	93	CL_{95}	0.0050	Dose
0.0060	100			0.0112
				(112 mg/L)

α-pinene				
0.0040	35	CL_{50}	0.0052	3.7
0.0050	46			Diagnostic
0.0060	56	CL_{95}	0.014	Dose
0.0070	67			0.044
0.0080	78			(440 mg/L)

Table 1. Concentrations used and rates obtained with derivatives of Pinaceae in *Cx quinquefasciatus* larvae.

Conc (%)	Mortality (%)	Lethal Conc		Slope of the Line
Chenopodium ambrosoide				
0.0040	10			11.8
0.0050	42	CL_{50}	0.0051	Diagnostic
0.0060	80			Dose :
0.0070	95	CL_{95}	0.0071	0.0162
0.0080	98			(162 mg/L)
Curcuma longa				
0.0040	46			5.22
0.0050	66	CL_{50}	0.0041	Diagnostic
0.0060	80			Dose :
0.0070	86	CL_{95}	0.0086	0.0232
0.0080	94			(232 mg/L)
Psidium rotundatum				
0.0040	28			8.16
0.0050	58	CL_{50}	0.0047	Diagnostic
0.0060	80			Dose
0.0070	92	CL_{95}	0.007	0.018
0.0080	97			(180 mg/L)
Piper aduncum				
0.0100	36			6.72
0.0120	53	CL_{50}	0.0110	Diagnostic
0.0140	73			Dose
0.0160	85	CL_{95}	0.0200	0.050
0.0180	90			(500 mg/L)
0.0200	95			
Pimenta racemosa				
0.0040	10			5.23
0.0050	20	CL_{50}	0.0070	Diagnostic
0.0060	44			Dose
0.0070	50	CL_{95}	0.0140	0.0380
0.0080	62			(380 mg/L)
0.0100	79			
Eugenia melanadenia				
0.0240	14			18.5
0.0260	41	CL_{50}	0.026	Diagnostic
0.0280	70			Dose
0.0300	78	CL_{95}	0.033	0.0718
0.0320	90			(718 mg/L)

Table 2. Concentrations used and rates obtained with essential oils tested in *Cx quinquefasciatus* larvae.

4. Conclusions

An interest in natural products from plants has been increased due to the development of resistance to synthetic insecticides, which are applied in order to reduce the populations of insects.

The biological activity of natural compounds is based on its structure and the doses used for such purposes. Vegetable insecticides have the great advantage of being compatible with other acceptable low-risk options from the ecological point of view on the control of insects. The secondary metabolites produced by plants against the attacks of predators and insects make them natural candidates in the control of species of insects, both vector of diseases and pests of agriculture. It is not logical to come to jump to the idea that they will completely replace the synthetic insecticides. Logical thinking is to have in them a complementary use to optimize and increase the sustainability of current integrated pest control strategies.

There are many publications of lists of plants with insecticidal properties. To use such plants, it is not enough to be regarded as promising or proven insecticidal properties. Analysis of risks to the environment and health should also be made. It is not to recommend the use of plants that are endangered, with limited biomass or that their use involves major alterations to the density in which they are in the nature. An ideal insecticide plant must be perennial, be widely distributed and in large amounts in nature or that can be cultivated, using renewable plant bodies such as leaves, flowers or fruits, not be destroyed every time you need to collect material to (avoid the use of roots and bark), agro-technitian minimum requirements and be eco-sustainability, have additional uses (such as medicines), not having a high economic value, be effective at low doses, possess potential scaling biotechnology (Silva;2002).

Insecticide plants have the advantage of having other uses as medicinal, a rapid degradation which decreases the risk of residues in food and therefore can be more specific for pest insect and less aggressive with natural enemies. They also develop resistance more slowly in comparison with synthetic insecticides. By the other hand, the disadvantages include that they can be degraded more quickly by ultraviolet rays so its residual effect is low, however not all insecticides from plants are less toxic than synthetic and residual is not established.

We can conclude that studies in our country, essential oils and their derivatives from the different families of evaluated plants have a high insecticide activity on larvae of *Aedes aegypti* and *Culex quinquefasciatus* and derived specifically from pinaceas, they have ovicide and inhibitory action of development in *Aedes aegypti*, which are potential candidates for control alternatives in these species of insects.

5. References

Adam, KP; Thiel, R; Zapp, J; Becker, H. (1998). Involment of the mevalonic acid pathway and glyceraldehyde-pyruvate pathway in the terpenoid biosynthesis of the liverworts *Ricciocarpos natansand Conocephalum conicum*. *Archives of Biochemistry and Biophysics*. 354: 181-187.

Aguilera, L; Navarro, A; Tacoronte, JE; Leyva, M ;Marquetti MC. (2003) Efecto letal de Myrtaceas cubanas sobre *Aedes aegypti* (Díptera: Culicidae) *Rev Cubana Med Trop*;55(2):100-4.

Aguilera L, Tacoronte JE, Navarro A, Leyva M, Bello A, Cabreras MT. (2004) Composición química y actividad biológica del aceite esencial de *Eugenia melanadenia* (Myrtales:

Myrtaceae) sobre *Blattella germanica* (Dictyoptera: Blattellidae). *Revista CENIC Ciencias Químicas*. : 35(3).

Alburquerque, MR; Silveira,ER; De Ochoa, DE; Lemus, TL; Souza, EB; Santiago, GM; Pessoa, OD. (2004) Chemical composition and larvicidal activity of the essential oils from *Eupatorium bentonicaea* Baker (Astaraceae) *J Agric Food Chem*.;52 (22): 6708-11.

Amer, A & Mehlhorn, H. (2006) Repellency effect of forty-one essential oils against *Aedes, Anopheles* and *Culex* mosquitoes. *Parassitol Res*.99 (4):478-90.

Amer, A & Methlhorn, H. (2006).Larvicidal effects of various essentials oils against, Aedes, Anopheles and Culex larvae (Diptera: Culicidae). *Parasitol Res*.99(4):466-72.

Ansari, MA; Mittal, PK; Razdan, RK; Sreehari, U. (2005) Larvicidal and mosquito repellent activities of Pine (*Pinus longifolia*, Familia: Pinaceae) oil. *J Vect Borne Dis*. 95-99.

Assarm, AA; El-Sobky, MM. (2003) Biological and histopathological studies of some plant extracts on larvae of *Culex pipiens* (Diptera:Culicidae) *J. Egypt Soc Parasitol l*.33(1):189-200.

Awde, JA; Ryan, MF (1992) Piperazine as a reversible competitive inhibitor of acetylcholinesterase. *Cienc. Biol.Ecol.Syst*. 12: 37-42.

Barnard, DR & Xue, RD. (2004) Laboratory evaluation of mosquito repellent against *Ae albopictus, Culex nigripalpus* (Diptera: Culicidae). *J Med Entomol*. 41 (4): 726-30.

Bassole, IH; Guelbeogo, WM; Nebie, R; Constantini, C; Sagnon, N; Kabore, Z; Traore, SA. (2003) Ovicidal and larvicidal activity against *Ae aegypti* and *An gambiae* complex mosquitoes of essential oils extracted from three spontaneus plants of Burkina Faso. *Parassitologia* 45 (1):23-6.

Bottia, E; Díaz, O; Mendivelso, D; Martínez, J; Stashenko, E. (2007) Comparación de la composición química de los metabolitos secundarios volátiles de cuatro plantas de la familia Piperaceae obtenidos por destilación–extracción simultánea. Scientia et Technica;13(33). ISSN 0122-1701

Bloomquist, J. (1999) Insecticides: Chemistries and Characteristics. Departmentof entomology. Virginia Polytechnic institute and state university. Blacksslomg Virginia. In http://ipmworld.umn.edu/chapters/bloomq.htm.

Byeoung-Soo, P; Won-Sik, C; Jeong-Han, K; Kap-Ho, K; SPNG-Eun, L. (2005). Monoterpenes from Thyme (Thymus vulgaris) as potencial mosquito repellents. *J Am Mosq. Control Association* .21 (1);80-83.

Caballero-Gallardo, K; Olivero-Verbel, J; Stashenko, EE.(2011) Repellent Activity of Essential Oils and Some of Their Individual Constituents against *Tribolium castaneum* Herbst.*J Agric Food Chem*. Feb 3. [Epub ahead of print]

Carvalho, AF; Melo, VM; Carveiro, AA; Machoo, MI; Beantim, MB; Rabelo, EF. (2003) Larvicidal activity of the essential oil fro *Lippia sidioide* against *Aedes aegypti*. *Mem Oswaldo Cruz*.98 (4): 569-71.

Cavalcanti, ES; Morais, SM; Lima, MA; Santana, EW. (2004) Larvicidal activity of essentials oils from Brazilian plants against *Ae aegypti*. *Mem Inst Oswaldo Cruz*.99(5):541-4.

Cetin H, Yani A. Study of the larvicidal activity of Origanum (Labiatae) species from Southwest Turkey. *J. Vector Ecol.* 2006; 31 (1): 118-22.

Chaiyasit, D; Choochate, W; Rattanachanpichai, E; Chaithong, U; Chaiwong P et al. (2006) Essential oils as potencial adulticides against two populations of *Aedes aegypti*, the laboratory and natural field strain in Chiang Mai province northern Thailand. *Parasitol Res* 1

Chang, KS; Talk, JH; Kim, SI; Lee, WJ; Ahn, YJ. (2006) Repellency of *Cinnamomum cassia* bark compounds and cream countain cassia oil to de *Ae aegypti* under laboaratory and indoor conditions. *Pest Manag Sci*. 62 (11):1032-1038.

Choochate, W; Chaiyasit, D; Kanjarapotti, D; Rottana, E; Champiochi, E et al (2005). Chemical composition and antimosquito potencial of rhizome extract and volatile oil derivade from *Curcuma aromatica* against *Aedes aegypti* (Diptera: Culicidae). *J Vector Ecol*.30 (2):302-9.

Champakaew, D; Choochote, W; Pongpaibul, Y; Chaithong, U; Jitpakdi, A; Tuetun, B; Pitasawat, B. (2007) Larvicidal efficacy and biological stability of a botanical natural product, zedoary oil-impregnated sand granules, against *Aedes aegypti* (Diptera, Culicidae). *Parasitol Res*.100(4):729-37.

Chansang, U ; Zahiri ,NS ; Bansiddhi, J ; Boonruad, T ; Thongsrirak, P ; Mingmuang, J ; Benjapong , N ; Mulla, MS. (2005) Mosquito larvicidal activity of aqueous extracts of long pepper (*Piper retrofractum* vahl) from Thailand. *J Vector Ecol*.30(2):195-200.

Cheng, SS; Liu, JY; Tasai, KH ; Chen, WJ; Chang, ST. (2004) Chemical composition and mosquito larvicidal activity of essential oils from leaves of different *Cinnamomum osmophloem* provenanaces. *J Agric Food Chem*.52 (14):4395-400.

Chung, IM; Song, HK; Yeo, MA; Moon, HI. (2010) Composition and immunotoxicity activity of major essential oils from stems of *Allium victorialis* L. var. platyphyllum Makino against *Aedes aegypti L. Immunopharmacol Immunotoxicol*. Dec 16. [Epub ahead of print]

Chung, IM; Ro, HM; Moon, HI (2010) Major essential oils composition and immunotoxicity activity from leaves of *Foeniculum vulgare* against *Aedes aegypti* L. *Immunopharmacol Immunotoxicol*. Nov 16. [Epub ahead of print]

Corbino, G . (2000) Productos naturales vegetales de interés económico Instituto Nacional de Tecnología Agropecuaria. Estación Experimental San Pedro. Buenos Aires, Argentina.Lab.Prod.InVitro.(11-2-2005)In : http://www.inta .gov.ar/sanpedro /1_sala_de_lectura/difusion/novedades.../corbinocondim.ht.

Das, NG; Barauah, I; Talukdar, PK; Das, SC. Evaluation of botanicals as repellent against mosquitoes. *J Vect Borne Dis* ;40 (1-2): 49-53.

De Mendoca, FA; Da Silva, KF; Dos Santos, KK; Ribeneiro Junior, KA; Sant'Ana, AE. (2005) Activities of some brazilian plants against larvae of the mosquito *Ae aegypti*.*Fitoterapia*;76 (7-8):629-36.

Dharmagada, VS; Naik, SN; Mittal, PK; Vasudevan, P. (2005) Larvicidal activity of *Tagetes patula* essential oil against three mosquito species. *Bioresour Technol* 96 (11);1235-40.

Faley, DH & Frances, SP. (2005) Laboratory evaluation of coconut oil as a larvicide for *Anopheles faraoti* and *Culex annulirostris*. *J Am Mosq.Control Assoc*.;21(4): 477-9.

Gascón, AD; Pelayes, MA. (2002). *Generalidades sobre los procesos extractivos en la obtención de aceites esenciales* Departamento de Tecnología Agroindustrial Facultad de Ciencias Agrarias Chacras de Coria – Mendoza.

Gillij, YG ; Gleiser, RM ; Zygadlo, JA. (2008) Mosquito repellent activity of essential oils of aromatic plants growing in Argentina . *Bioresour Technol*.99(7): 2501-15.

González, P.(1984) Capítulo VI In "*Utilización Terapeútica de Nuestras Plantas Medicinales*", Universidad de La Salle, Bogotá.

Gunderson, CA; Samuelian, JH; Evans ,CK. (1985) Effects of the mint monoterpene pulegone on *Spodoptera eridinan* (Lepidoptera: Noctuidae). *Environ Entomol*.14:859-63.

Hafeez, F; Akram, W; Shaalan, EA. (2011).Mosquito larvicidal activity of citrus limonoids against *Aedes albopictus*. *Parasitol Res.* Jan 7. [Epub ahead of print]

http://www.buscasalud.com/boletín/analisis/2003_01_1117_09_32.html (12-1-2005)

http://www.chemkeys.com/esp/md/tee_6/ecoqui_2/metqui_5/metqui_5.htm (8-1-2007)

http://www.herbotecnia.com.ar/poscosecha-esencias.html (8-1-2007)

http://www.losandes.com.ar/imprimir.asp?nrc=163985 (8-1-2007)

http://www.cannabiscultura.com/f1/showthread.php?p=84457 (4-3-2011)

Ikan, R.(1991) Natural products. A laboratory guide. Second Edition. Academia Press (Eds).360 pp

Jaenson, TG; Palsson, K; Borg-Karlson, AK. (2006) Evaluation of extracts and oils of mosquito (Diptera: Culicidae) repellent plants from Sweden and Guinea Bissau. *J Med. Entomol.*43 (1):113-9.

Judd, WS; Campell, CS; Kellogg, EA; Stevens, PF; Donoghue, MJ. (2002) Secondary plants compounds. Plant systematic: a phylogenetic approach. Chapter 4, Structural and Biochemical Characters. Second Edition. Sinaeur Axxoc, USA.

Keane, S;Ryan, MF. (1999). Purification, characterization and inhabitation by monoterpenesof acetylcholinesterase from the waxmoth *Galleria mellonella*. *Insect Biochemistry and Molecular Biology* 29:1097-1104.

Khandagle, AJ; Tare, VS; Raut, KD; Morey, RA. (2011).Bioactivity of essential oils of *Zingiber officinalis* and Achyranthes aspera against mosquitoes. *Parasitol Res*. Feb 11. [Epub ahead of print]

Kim, SI; Chang, KS; Yang, YC; Kim, BS; Ahn, YJ. (2004) Repellency of aereosol and cream products containg fennel oil to mosquitoes under laboratory and field conditions. *Pest Manang Sci*.;60 (11):1125-30.

Kordali, S; Cakir, A; Ozer, H; Cakmakci, R; Kesdek, M; Mete, E. (2008)Antifungal, phytotoxic and insecticidal properties of essential oil isolated from Turkish *Origanum acutidens* and its three components, carvacrol, thymol and p-cymene. *Bioresour Technol*. May 28.

Kostyukovsky, M; Rafaeli, A; Gileadi, C; Demchenko, N; Shaaya, E. (2002). Activation of octopaminergic receptors by essential oil constituent isolated from aromatic plants: posible mode of action against insect pest. *Pest Management Science*. 58: 1101-06.

Leyva M , Aguilera L, Tacoronte J, Montada D, Bello A, Marquetti MC. (2007) Estudio de laboratorio del aceite esencial de *Pimenta racemosa* (Myrtales: Myrtaceae) y su posible utilización para el control de *Musca domestica* (Diptera: Muscidae) *Rev CNIC Ciencias Químicas*

Leyva M, Tacoronte J, Marquetti MC.(2007) Composición química y efecto letal de *Pimenta racemosa* (Myrtales: Myrtaceae) en *Blatella germanica* (Dictyoptera: Blattellidae) Rev. Cub. Med. Trop . 59 (2)

Leyva M, Tacoronte JE, Marquetti MC, Scull R, Montada D, Rodríguez Y, Bruzón R.(2008)Actividad insecticida de aceites esenciales de plantas en larvas de *Aedes aegypti* (Diptera: Culicidae) *Rev Cubana Med Trop* 60(1):78-82.

Leyva M, Tacoronte JE,Marquetti MC, Montada D.(2008) Actividad insecticida de tres aceites esenciales en *Musca domestica* (Diptera: Muscidae) Rev Cubana Med Trop 60(3).

Leyva M, Tacoronte JE, Marquetti MC ,Scull R , Tiomno O , Mesa A, Montada D. (2009) Utilización de aceites esenciales de pinaceas endémicas como una alternativa de control en *Aedes Aegypti Rev .Cub . Med.Trop*;61(3):239-43.

Leyva M, Marquetti MC, Tacoronte JE, Scull R , Tiomno O , Mesa A, Montada D. (2009)Actividad larvicida de aceites esenciales de plantas contra *Aedes aegypti* (L) (Díptera: Culicidae) *Biomed* 20: 5-13.

Leyva M, Marquetti MC, Tacoronte JÁ, Tiomno O, Montada D (2010) Efecto inhibidor del aceite de trementina sobre el desarrollo de larvas de *Aedes aegypti* (Diptera: Culicidae) *Rev.Cub.Med.Trop*.;62(3):

López, MD.(2008) Toxicidad volatil de monoterpenoides y mecanismos bioquímicos en insectos plagas del arroz almacenado. Tesis Doctoral. Universidad de Murcia. Facultad de Quimica. España 245 pp

Lucia, A; Gonzalez Audino, P; Saccacini, E; Licastro, S; Zerba, E; Masuh, H (2007).Larvicidal effect of *Eucalyptus grandis* essential oil and turpentine and their major components on *Ae aegypti* larvae *J.Am.Mosq Control Assoc.*23:293-303

Mathew, J; Thoppil, JE.(2011) Chemical composition and mosquito larvicidal activities of Salvia essential oils. *Pharm Biol*. Feb 2. [Epub ahead of print]

Miot, HA; Batistilla, RF; Batista, K; de A Volpato, DE, Augusto, LS; Madeira, NG; Haddad, V; Miot, LD. (2004) Comparative study of the topical effectiveness of the Andiroba oil (*Coparais guianensis*) and DEET 50 % as repellent for Ae sp. *Rev Inst Med. Trop Sao Paaulo*.46 (5): 253-6.

Morais, SM; Cavalvanti, ES; Bertini, LM; Oliveira, CL; Rodríguez, JR; Cardoso, JH. (2006) Larvicidal activity of essential oils from Brazilian Croton sepecies against *Ae aegypti*. *J Am Mosq. Control Assoc*. 22(1):161-4.

Odalo, JO; Omolo, MO; Malebo, H; Angira, J; Njeru, PM; Ndiege, IO; Hassanali ,A. (2005) Repellency of esential oils of some plants from Kenya coast against *An gambiae*. *Acta Tropical*.95 (3):210-8.

Oyedale, AO; Gbolade, AA; Sosan, MB; Adewoyin, FB; Sayelu, OL; Orafidaya, OO. (2002) Formulation of an effective mosquito-repellent topical product from lemon gross. *Phytomedicine*.9(3):259-62.

Parmar, V.S; Jain, S.C; Bisht, K.S; Jain, R; Taneja, P; Jha, A ;et al. (1997) Phytochemistry of the genus *Piper*. *Phytochemistry*. 46:597-673

Pérez-Pacheco, R; Rodríguez –Hernández, C ; Lara-Reyna, J; Montes –Belmont, R; Ramírez – Valverde, G. (2004) Toxicidad de aceites, esencias y extractos vegetales en larvas de mosquito *Culex quinquefasciatus* Say (Diptera: Culicidae) *Acta Zoológica Mexicana*. 20(1): 141-152.

Phasomkusolsil, S; Soonwera, M. (2010) Potential larvicidal and pupacidal activities of herbal essential oils against *Culex quinquefasciatus* say and *Anopheles minimus* (theobald).*Southeast Asian J Trop Med Public Health.* Nov;41(6):1342-51.

Phasomkusolsil, S; Soonwera, M. (2010) Insect repellent activity of medicinal plant oils against *Aedes aegypti* (Linn.), *Anopheles minimus* (Theobald) and *Culex quinquefasciatus* Say based on protection time and biting rate.*Southeast Asian J Trop Med Public Health*. Jul;41(4):831-40.

Pino, J. Borges P. (1999) Los componentes volátiles de las especias. I. Métodos de obtención y de análisis. *Alimentaria*.30 (1) 39-45.

Pino, JA; Marbot, R; Bello, A; Urquiola, A (2004) Essential Oils of *Piper peltata* (L.) Miq. and *Piper aduncum* L. from *Cuba Journal of Essential Oil Research: JEOR*, Mar/Apr 2004

Pino, J A; Marbor, R; Bello, A;Urquiola, A. (2005) Essential oil of *Eugenia melanadenia* Krug et Urb. from Cuba". *Journal of Essential Oil Research* JEOR. FindArticles.com. (09-2-2011) In: . http://findarticles.com/p/articles/mi_qa4091/is_200307/ai_n9287900/

Pitarokili, D; Michaelakis, A; Koliopoulos, G; Giatropoulos, A; Tzakou, O. (2011). Chemical composition, larvicidal evaluation, and adult repellency of endemic Greek Thymus essential oils against the mosquito vector of West Nile virus. *Parasitol Res*. Feb 8.

Pohlit, AM; Lopes, NP; Gama, RA; Tadei, WP; de Andrade-Neto, VF. (2011) Patent Literature on Mosquito Repellent Inventions which Contain Plant Essential Oils - A Review.*Planta Med* Feb 15. [Epub ahead of print]

Prajapati, V; Tripathi, AK; Aggarwall, KK; Kanuja, SP (2005) Insecticidal repellent and oviposition-deterrente activity of selected essential oils against *An stephensi, Ae aegypti* and *Cx quinquefasciatus*. *Bioresour Technol*. 96 (16): 1749-57.

Pushpanathan, T; Jebanesan, A; Govindarajan, M. (2006) Larvicidal, ovicidal and repellent activities of *Cymbopogan citratus* Stapf (Graminae) essential oil against the filarial mosquito *Culex quinquefasciatus* (Say) (Diptera : Culicidae). *Trop Biomed*. 23(2):208-12.

Ravi, K; Bhavani, K; Sita Devi, P; Rajaswara Rao, BR; Janarahan, RK. (2006) Composition and larvicidal activity of leave and stem essential oil of *Chloroxylon swetenia* against *Ae aegypti* and *An stephensi. Bioresour Technol.* 97(18):24-31.

Ryan, MF; Byrne, O (1988) Plant-insect coevolution and inhibition of acetilcholinesterase. *Journal of Chemical Ecology* 14:1965-75.

Ryan, MF; Awde, J; Moran, S. (1992) Insect pheromones as reversible competitive inhibitors of acetilcholinesterase. *Invertebrate reproduction and development*. 22:31-38.

Scholes, M. (1995). *La Terapia de los Aromas*. Sterling Press, New York.

Stamm, M. D. (1970).Modernos Conocimientos sobre la Química de los Aceites Esenciales. Métodos de Análisis. *C.S.I.C. Manuales de Ciencia Actual 3*. Patronato "Alonso de Herrera", Madrid.

Sangkyun L; Tsao, R; Coats, J. (1999) Influence of dietary applied monoterpenoids and derivates on survival and growth of european corn borer (Lepidoptera: Pyralidae). *J Econ Entomol*.92(1):56-67.

Smith, RM & Kassim, H. (1979) The essential oil of *Piper aduncum* from Fiji. New Zealand. *J Sci* . 22:127-128.

Sponsel VM. (1995). The biosyntesis and metabolism of gibberellins in higher plants. In Plant hormones. Davies PJ (Ed). Kuwer Academic Publishers Boston. 66-97 pp.

Suparvarn, P; Knapp, F; Sigafus, R. (1974) Biologically active plant extracts for control of mosquito larvae. *Mosquito News*.34(4):398-402

Silva, WJ; Dória, GA; Maia, RT; Nunes, RS; Carvalho, GA; Blank, AF; Alves, PB; Marçal, RM; Cavalcanti, SC. (2008) Effects of essential oils on *Aedes aegypti* larvae: Alternatives to environmentally safe insecticides. *Bioresour Technol.*;99(8):3251-5

Silva, G. (2002) *Insecticidas vegetales*. (11-12-2006) In: http://ipmworld.umn.edu/ cancelado /Spchapters/GSilvaSp.htm

Silva, G.(2003) Resistencia a los insecticidas In: Silva, G y Hepp, R (Eds). Bases para el manejo racional de insecticidas. Universidad de Concepción, Fundación para la Innovación Agraria: 237-60pp

Stashenko, E. (1996) In: *Memorias del IV Congreso Nacional de Fitoquímica*, Universidad Industrial de Santander, Escuela de Química, Bucaramanga, febrero de 1996, pp. 29-53.

Tare, V; Deshpade, S; Sharma, RN. (2004) Susceptibility of two different strains of *Ae aegypti* (Diptera: Culicidae) to plants oils. *J Econ. Entomol*. 97 (5):1734-6.

Tawatsin, A; Asavadachanukorn, P; Tahavara, U; Wongsenkongman, P; Bansidhi, J; Boonroad, J. (2006) Repellency of essential oils extracted from plantas in Thailand against four mosquito vectors (Diptera: Culicidae) *Southeast Asian J Med Trop. Med Public Heath*. 37 (5):915-31.

Thomas, TG. (2004) Mosquito larvicidal properties of essential oil of indigenus plant *Ipomoea airica* L. *Jpn J Infect Dis*.57 (4).

Trongotokit, Y; Curtis, CF; Rangsriyum, Y. (2005) Efficacy of repellent products against caged and free flying *An stephensi* mosquitoes Southeast Asian. *J Trop Med. Public Health*. 36 (6): 1423-31.

Trongotokit, Y; Rangsriyum, Y; Komalamisra, N; Apiwathnarson, C. (2005) Comparative repellency of 38 essential oils against mosquito bites. *Phytother Res*.; 19(4):303-9.

Worwood, V. (1992) *The Complete Book of Essential Oils and Aromatherapy*. New World Library, New York.

Yang, P& Ma, Y. (2005) Repellent effects of plants essential oils against *Ae albopictus*. *J Vector Ecol*. 30 (2): 231-4.

Zhu, J; Zeng, X; Yanma, J; Liu, J; Qian, K; Tucker, B; Schultz, G; Coats, J; Zang, A. (2006) Adults repellency and larvicidal activity of five planta essential oil against mosquitoes. *J Am Mosq Control Assoc* 22(3):515-22.

11

Biological Control of Mosquito Larvae by *Bacillus thuringiensis* subsp. *israelensis*

Mario Ramírez-Lepe[1] and Montserrat Ramírez-Suero[2]
[1]*Instituto Tecnológico de Veracruz, UNIDA, Veracruz,*
[2]*Université de Haufe-Alsace, LVBE EA-3991, Colmar,*
[1]*Mexico*
[2]*France*

1. Introduction

Chemical insecticides provide many benefits to food production and human health and has proven very effective at increasing agriculture and forestry productivities. However, they also pose some hazards as contamination of water and food sources, poisoning of non-target fauna and flora, concentration in the food chain and selection of insect pest populations resistent to the chemical insecticides (Wojciech & Korsten 2002). It is well documented that chemical pesticides reduced natural-enemy populations and chemical applications can disrupt biological control and may cause outbreaks of secundary pests previously suppressed by natural enemies (Bartlett, 1964) and pest species develop pesticide resistance but natural enemies not (Johnson & Tabashnick, 1999).

The use of synthetic organic pesticides has had serious economic, social and environmental ramifications. Economically, the rapidy increasing cost for development and production of petrochemically derived insecticides, together with the declining effectiveness due to widespread insect resistance. As a result the chemical pesticide industry continues to develop new more expensive compounds and increasing pesticide prices. Socially and ecologically they have caused death and disease in human and damaged the environment. It is estimated that only a minute fraction of the insecticides applied is required for suppression of the target pest. The remainder, more than 99.9%, enters the environment through soil, water and food cycles (Metcalf, 1986).

Alternative methods of insect management offer adequate levels of pest control and pose fewer hazards. One such alternative is the use of microbial insecticides, that contain microorganisms or their by-products. Microbial insecticides are especially valuable because their toxicity to non-target animals and humans is extremely low. Compared to other commonly used insecticides, they are safe for both, the pesticide user and consumers of treated crops. Microbial insecticides also are known as biological pathogens, and biological control agents. Chemical insecticides are far more commonly used in the world than microbial control, however some microbial control agents, at least in part, can be used to replace some hazardous chemical pest control agents. A number of biological control agents formulated with bacteria, fungi, virus, pheromones, and plant extracts have been in use mainly for the control of insects responsible for the destruction of forests and agriculture crops (McDonald & Linde, 2002).

The microbial insecticides most widely used in the world are preparations of *Bacillus thuringiensis* (*Bt*). The insecticidal activity of *Bt* is due to the proteic parasporal inclusions that are produced during sporulation. Insecticides based on the proteinaceous δ-endotoxin of *Bt* constitute part of a more ecologically rational pest control strategy. *Bt* strains have been isolated world wide from many habitats, including soil, insects, stored-product dust, and deciduous and coniferous leaves, all of which have a limited host range, however together span a wide range of insects orders which include: Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Phthiraptera, Orthoptera, Acari, and Mallophaga and other organisms such as nematodes, mites, and protozoa (Federici, 1999). *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) or serotype H-14, exhibit acute toxicity towards dipteran insects such as larval mosquitoes and black flies (de Barjac 1978) and is currently used in mosquito control programs world wide (Priest, 1992). The World Health Organization's (WHO) Onchocerciasis Control Program (OCP) in West Africa using *Bti* toxins has been one of the success stories of international co-operation in the control of infectious diseases program (Webb 1992; Drobniewski 1993). Due to the importance of *Bti* to control several tropical diseases such malaria and dengue, our purpose in this chapter is to provide an overview for non-*Bt* specialists of the basic knowledge of *Bti.*

2. *Bacillus thuringiensis* strains

2.1 Origin of some strains of *B. thuringiensis*

A large number of strain of *Bt* have been isolated from which to date. *Bt* as currently recognized is actually a complex of subspecies. They have grouped in 79 serotypes (Zeigler, 1999). The first *Bt* strain was isolated from diseased larvae of the silkworm, *Bombi mori*, in Japan by Ishiwata (1901). Iwabushi (1908) describe the bacillus as *Bacillus sotto.* Aoki & Chigasaki (1915) and Mitani & Wataral (1916) purified a highly toxic substance from sporulated *Bt* cultures. It was not officially described, however, until it was reisolated by Berliner in 1915 from diseased larvae of the Mediterranean flour moth, *Anagasta kuehniella*, in Thuringia, Germany, hence the derivation of species name thuringiensis (Federici, 1999). A *Bt* strain was isolated again by Mattes (1927) and described briefly the inclusion rhomboidal body. The activity of the *Bt* strains against lepidopteran larvae was described by Metalnikov (1930) and by Husz (1931). The association of the inclusion bodies of *B. thuringiensis* with toxicity against insects was established by Steinhaus. In 1951 published a paper which described the morphology of *Bt* and its possible use in the biological control against the alfalfa caterpillar. In France, a product named "Sporeine" was developed and used against *Ephestia kuhniella* (Lepidoptera) (Jacobs, 1950). Hannay, (1953) described a parasporal body in bipyramidal shape produced by the bacterium during sporulation and suggested that the crystal was involved in the toxic activity. The protein nature of the crystals was determined by Hanna and Fitz-James (1955).

2.2 Features of *B. thuringiensis* strains

Bt is a facultative anaerobic, gram-positive bacterium that forms characteristic protein inclusions adjacent to the endospore (Fig.1). The crystalline inclusion are composed of proteins known as ICPs crystal proteins. Cry proteins, or δ-endotoxins is the basis for commercial insecticidal formulations of *Bt.* Insecticides containing *Bt* in pest control programs is now considered as a viable strategy, which has proven to be both safe and reliable over the last 45 years (Chungjatupornchai et al. 1988).

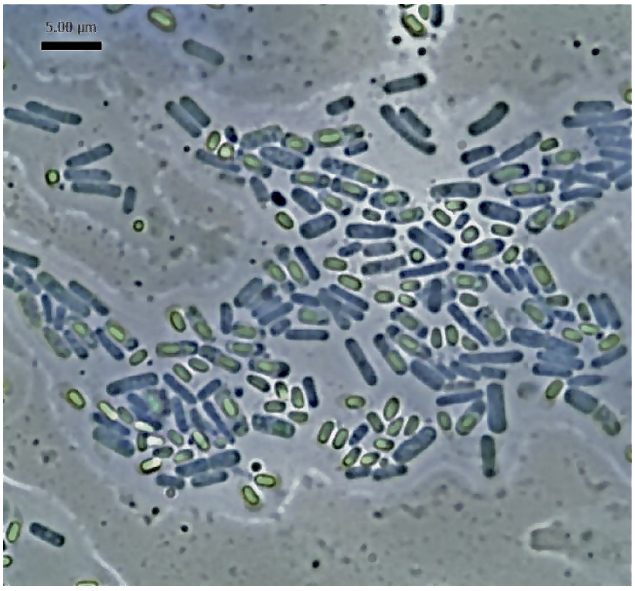

Fig. 1. *Bacillus thuringiensis* subsp. *israelensis.*

2.3 Advantages and disadvantages of *Bt*

According to Federici (1999) the main reasons for the success of *Bt* include (i) the high efficacy of its insecticidal proteins (ii) the existence of a diversity of proteins that are effective against a range of important pests (iii) its relatively safety to nontarget insect predators and parasites (iv) its easy to mass production at a relatively low cost, and (v) its adaptability to conventional formulations and application technology.

Advantages and disadvantages of *Bt* with chemical insecticides were summarized by Rowe and Margaritis (1987).

Advantages of *Bt:*

- High specificity, i.e. no mammalian or nontarget effects; use permitted up to date of harvest.
- No broad insect resistance observed or expected to develop.
- Adaptable to many types of formulations; potential to incorporate feeding stimulants or baits to increase the attractiveness of the formulations to the insects and thereby increase their efficacy.
- Probability of producing more potent formulations and reducing production costs through improved-fermentation technology.
- High probability that strain selection and/or genetic engineering will lead to better control of pest insects by newly found or created strains of Bt having novel host spectra or increased activity

Disadvantages of *Bt:*

- Narrow host spectrum
- Lack of patent protection on new strains
- Proper timing of application require due to slower effect than chemical insecticides

- Activity dependent of ingestion, and feeding activity depends on environmental conditions
- Relatively higher cost compared to chemical insecticides

2.4 Clasification of strains of *Bacillus thuringiensis*

Bt is a crystalliferous spore-forming bacterium close genetic relationship with *B. cereus, B. anthracis* and *B. mycoides* (Höffe & Whiteley, 1989). The classification of *Bt* is difficult because DNA sequencing studies of conserved gene regions of theses species have suggested that they belong to a single specie. *Bt* strains are distinguished from *B. cereus, B. anthracis* and *B. Mycoides* by the ability to produce parasporal crystalline inclusions during sporulation (de Barjac, 1978). Crystal formation is the criterion for distinguishing between *B. cereus* and *Bt*, otherwise they could be considered as the same specie. Research based on a comparative study of 16s rRNA sequences, *Bt* and *B. cereus* var. *mycoides* differed from each other and from ***B.*** *anthracis* and *cereus* by less than nine nucleotides (Ash and Collins 1992). Chen & Tsen (2002) amplified 16S rDNA and *gyrB* gene by PCR and they found that the discrimination between *B. cereus* and *Bt* strains, when a large number of Bacillus strains were tested was difficult. They proposed, to distinguish *Bt* from *B. cereus*, a single feature, such as the presence of a parasporal crystal protein or cry gene is reliable.

Several attemps were made to classify *Bt* strains. de Barjac and Frachon (1990) didn´t find correlation between biochemical reactions and 27 H serotypes using 1,600 *B. thuringiensis* isolates. They demonstrated that the current biochemical tests have no value as the sole criteria for differentiating *Bt* strains. Another approach ot these authors for classification was the use of susceptibility to certain bacterial viruses called phages. There are 14 bacteriophages that have been used for *Bt* but phage typing is inconsistent with serotyping and does not permit classification. They found frequent cross-reactions.

One widely clasification system for *Bt* strains is based in the determination of the H-flagellar antigen technique described by Barjac & Bonnefoi (1962). This technique needs very motile bacterial cultures to prepare flagellar suspensions. These suspensions are titrated against antisera directed against *B. thuringiensis* strains of each serotype. Presently *B. thuringiensis* strains are classified within 79 serotypes. Table 1 shows the classification of *Bt* strains by serotypes.

Bt strains that had discovered previously to 1977 were pathogens towards lepidopteran larvae. However, in 1975 was discovered *B. thuringiensis* serovar *israelensis* toxic to mosquito larvae (Goldberg & Margarit 1977) and in 1983 a strain from *Bt* serovar *morrisoni* was found to be pathogenic to Coleoptera larvae (Krieg et al. 1983). According to these findings, serological clasification, although still in use as a basic method to clasify *Bt* strains could not be related with pathogenicity. Subsequently studies showed that, within a serotype, different activity, spectra can be found. For example, some strains of *B. thuringiensis* serotype *morrisoni* in their parasporal inclusión bodies contain different proteins and have activity against Diptera, Coleoptera or Lepidoptera.

With the knowledge of sequence of the genes that encode the proteins cry it was proposed a classification based on the cry toxin genes. Höfte and Whiteley (1989) proposed a nomenclature clasification scheme for *Bt* crystal proteins based in their structural aminoacid sequence, deduced from the DNA and host range. They named *cry* (crystal protein) genes and their related proteins, "Cry proteins". They clasified 42 *Bt* crystal protein genes into 14 distinct genes grouped into 4 major clases. The classes were CryI (Lepidoptera-specific), CryII (Lepidoptera-and Diptera-specific), CryIII (Coleoptera-specific), and CryIV (Dipter-specific).

Serotype	Serovar	Serotype	Serovar
1	thuringiensis	28a, 28c	jeghatensan
2	finitimus	29	amagiensis
3a, 3b, 3c	kurstaki	31	toguchini
3a, 3c	alesti	32	cameroun
3a, 3d	sumiyoshiensis	33	leesis
3a, 3d, 3e	fukuokaensis	34	konkukian
4a, 4b	soto/dendrolimus	35	seoulensis
4a, 4c	kenyae	36	malayensis
5a, 5b	galleriae	37	andralousiensis
5a,5c	canadensis	38	owaldocruzi
6	entomocidus/subtoxicus	39	brasilensis
7	aizawai/pacificus	40	huazhongensis
8a.8b	morrisoni	41	sooncheon
8a, 8c	ostriniae	42	jinghongiensis
8b, 8d	nigeriensis	43	guiyangiensis
9	tolworthi	44	higo
10a, 10b	darmstadiensis	45	roskildensis
10a, 10c	londrina	46	chanpaisis
11a, 11b	toumanoffi	47	wratislaviensis
11a, 11c	kyushuensis	48	balearica
12	thompsoni	49	muju
13	pakistani	50	navarrensis
14	israelensis	51	xiaguangiensis
15	dakota	52	kim
16	indiana	53	asturiensis
17	tohokuensis	54	poloniensis
18a, 18b	kumamtoensis	55	palmanyolensis
18a, 18c	yosoo	56	rongseni
19	tochigiensis	57	pirenaica
20a, 20b	yunnanensis	58	argentinensis
20a, 20c	pondicheriensis	59	iberica
21	colmeri	60	pingluonsis
22	shanongiensis	61	sylvestriensis
23	japonensis	62	zhaodongensis
24a, 24b	neolonensis	64	azorensis
24a, 24c	novosibirsk	65	pulsiensis
25	coreanensis	66	graciosensis
26	silo	67	vazensis
27	mexicanensis	none	wuhanensis
28a, 28b	monterrey		

Table 1. Classification of *B.thuringiensis* strains by serotype (modified after Zeigler, 1999).

The *cryI* genes can be distinguished from the other *cry* genes simply by sequence homology (>50% aminoacid identy) and encode 130 to 140 kDa proteins which accumulate in

bypiramidal crystalline inclusions during the sporulation of *Bt*. *cryII* genes encode 65-kDa proteins which form cuboidal inclusions in strains of several species. *cryIII* genes produces romboidal crystals containing one major protein, a 72kDa protein. cryIV class genes (*cryIVA*, cryIVB, cryIVC, and cryIVD) as well as *cytA* were all isolated from the same 72-Mda plasmid present in *Bt* subsp. *israelensis.*

3. *Bacillus thuringiensis* subsp. *israelensis* as an important part of mosquito control

3.1 Properties of *Bti*

In 1975-76 under a World Health Organization sponsored project, a new *Bt* strain was discovered in the Negev desert in Israel by Goldberg and Margalit (1977). The strain was isolated from *Culex* sp. dead larvae mosquito. Later was identified as *Bt israelensis*, serotype H14 according to its fagellar antigenicity by de Barjac (1978).

Bti has all the features taxonomic, morphological, growth, sporulation, of isolation, cultivation of other varieties of *B. thuringiensis* (Fig.1). The larvicidal activity of *Bt israelensis* on mosquito transmitted diseases was the most important feature of the strain. The insecticidal properties of this bacteria are due primarily to insecticidal proteins produced during sporulation. The key proteins are Cyt1A(27.3 kDa), Cry4A (128 kDa), Cry4B (134 kDa) and Cry11A (72 kDa) and in three different inclusion types assembled into a spherical parasporal body held together by lamellar envelope (Ibarra & Federici, 1986). The inclusions are realtively small (0.1 to 0.5μm) and there are usually two to four inclusions per cell which vary in shape from cuboidal to bipyramidal, ovoid or anamorph (Charles & de Barjac 1982; Mikola *et al.* 1982; Yamamoto *et al.* 1983)

3.2 Mosquitoes, important vectors of tropical diseases

Mosquitoes are important vectors of several tropical diseases that suck blood from human and animals. They are vectors of multiple of diseases of man through transmision of pathogenic viruses, bacteria, protozoa and nematodes (Priest, 1992). From the medical point of view, mosquitoes are among the most important insects due their capacity to transmit human diseases such as malaria and dengue.

Vector	Disease
Anopheles	Malaria, lymphatic filariasis
Culex	Lymphatic filariasis, Japanese encephalitis, other viral diseases
Aedes	Yellow fever, dengue, dengue hemorrhagic fever, other viral diseases, lymphatic filariasis
Mansonia	Lymphatic filariasis

Table 2. Some diseases transmitted by mosquito (Rawlins, 1989; Walsh 1986).

There are about 3000 species of mosquito, of which about 100 are vectors of human diseases. Some of the more important of these diseases are listed in Table 2. It is estimated two billion people worldwide living in areas where these are endemic (World Health Organization, 1999). Thus, there is an urgent need for new agents and strategies to control these diseases.

3.3 Susceptibility of mosquito species to *B. thuringiensis* serotype *israelensis*

Bti is highly pathogenic against *Culicidae* (mosquitoes) and *Simuliidae* (blackflies), and has some virulence against certain others Diptera, especially Chironomidae (midges). Mosquito have four distinct stages in their life cycle: egg, larva, pupa and adult. Depending on the specie a female lays between 30 and 300 eggs at a time on the surface of the water, singly (*Anopheles*), in floating rafts (*Culex*) or just above the water line or on wet mud (*Aedes*). Once hatched the larvae grow in four different stages (instars). The first instar measures 1.5 mm in length, the fourth instar about 8-10 mm. The fully grown larvae then changes into a comma shaped pupa. When mature, the pupal skin splits at one end and a fully developed adult emerges. The entire period from egg to adult takes about 7-13 days under good conditions (Wada, 1989). First instar is more suceptible to *Bti* than fourth instar (Mulla *et al.* 1990). Pupa does not feed and therefore is not affected by *Bti*. For almost all species tested, increasing age of the larvae resulted in reduced susceptibility in mosquito (Chen et al. 1984: Mulla *et al.* 1985). *Bti* was found to be specific toxic to larvae of 109 mosquito species (Table 3).

Mosquito genus	Species
Aedes	40
Anopheles	27
Culex	19
Culiseta	5
Mansonia	5
Psorophora	3
Armigeres	3
Toxorhynchites	2
Limatus	2
Trichophospon	1
Uranotaenia	1
Tripteroides	1
	Total 109

Table 3. Larvicidal activity of *Bti* on mosquito species (Glare & O´Callaghan 1998).

Among mosquitoes, different preparations of *Bti* have shown different levels of toxicity to host species. Others factors influencing the susceptibility of mosquito larva to *Bti*. For example, the effect of a given dosage of toxin could produce different results depending on weather the lethal dose is administered all at once or in same doses over a long period (Aly et al. 1988).

Bti has an LC50 in the range of 10–13 ng/ml against the fourth instar of many mosquito species (Federici *et al.* 2003). Generally, *Culex* and *Aedes* are highly susceptible while *Anopheles* are less suceptible, but can be killed with *Bti* (Balaraman *et al*. 1983). Much higher concentrations of *Bti* are required to induce mortality in anopheline larvae than in *Aedes aegypti* larvae (Goldberg & Margalit 1977; Nugud & White 1982)

The exception is *A. franciscanus* that is as susceptible as other genera (García *et al.* 1980, Sun *et al.* 1980). Furhtermore, even within one genus, some species are more suceptible than others (Chui et al. 1993). Sun et al. (1980) suggested that a difference in feeding behavior might account for differences in susceptibilities. Filtering rates vary between genera and species. For example Aly (1988) showed that in the absence of *Bti*, larvae cleared the suspensions with constant relative filtration rates of 632 (*Ae. aegypti*), 515 (*Cx. quinquefasciatus*) or 83.9

μL/Larvae/h (*An. albimanus*). Another factor to be considered for the suceptibility to *Bti* is the behavior of the larvae. *Anopheles* larvae filter-feed on food particles present at the surface of water or a few centimeters below it, where as *Culex* and *Aedes* larvae not only feed faster but are capable of filter-feeding at much deeper water dephts (Aly et al. 1988).
Mosquito species which are not filter feeders do not seem suceptible. For example, against *Culicoides occidentalis* (Colwell 1982) and *Coquillettidia perturbans*, *Bti* larvicides had no effect (Walker et al 1985). Solubilization of the protein reduced the toxicity dramatically (50- to -100 fold) this is attributed to reduced level of toxin ingestion by larvae owing to their filter feeding behaviour (Chungjatupornchai et al. 1988).

3.4 Susceptibility of mosquito species to purified *Bt* proteic crystals

Several efforts have been made to purify the proteins Cry of *Bti* and other *Bt* subps. with the purpose of studying the chemistry of Cry proteins, the synergism between them, and the effect of each crystal on larvae of different mosquito species. It is somewhat difficult to separate the *Bt* spores and crystals because they are of similar size and surface characteristics. For that reason several methods have been used to purify *Bt* crystals proteins. Using NaBr gradients (Chang et al. 1992), sucrose gradients (Debro et al. 1986), renografin gradients (Aronson et al. 1991), and in a separatory funnels (Delafield et al. 1968). Bioassays of purified Cry proteins have been allowed to know that not only *Bti* Cry proteins have activity on mosquito larva. Other purified Cry proteins of other *Bt* strains have also activity on mosquito larvae. Table 4 shows the mosquito toxicity of purified crystals of some *Bt* strains.

Name	Source Strain	Mosquito toxicity
Cry4Aa1	*B.t. israelensis* 4Q2-72	*Aedes agypti, Anopheles stephensi*, *Culex pipiens* (Diptera: Cuclidae)
Cry4Ba1	*B.t. israelensis* 4Q2-72	*Aedes agypti* (Diptera: Cuclidae)
Cry10Aa1	*B.t. israelensis* ONR60A	*Aedes agypti,* (Diptera: Cuclidae)
Cry11Aa1	*B.t. israelensis* HD-567	*Aedes agypti, Anopheles stephensi*, *Culex pipiens* (Diptera: Cuclidae)
Cry11Ba1	*B.t. jegathensan* 367	*Aedes agypti, Anopheles stephensi*, *Culex pipiens* (Diptera: Cuclidae)
Cry11Bb1	*B.t. medellin*	*Aedes agypti, Anopheles albimanus*, *Culex quinquefasciatus* (Diptera: Cuclidae)
Cry16Aa1	*Clostridium bifermentans malasya* CH18	*Aedes agypti, Anopheles stephensi*, *Culex pipiens* (Diptera: Cuclidae)
Cry19Aa1	*B.t. jegethesan*	*Anopheles stephensi*, *Culex pipiens* (Diptera: Cuclidae)
Cry20Aa1	*B.t. fukuokaensis*	*Aedes agypti,* (Diptera: Cuclidae)
Cry21Aa1	*B.t. higo*	*Culex pipiens molestus* (Diptera: Cuclidae)
Cyt1Aa1	*B.t. israelensis* IPS82	*Aedes agypti, Anopheles stephensi*, *Culex pipiens* (Diptera: Cuclidae)
Cyt1Ab1	*B.t. medellin* 163-131	*Aedes agypti, Anopheles stephensi*, *Culex pipiens* (Diptera: Cuclidae)
Cyt2Aa1	*B.t. kyushuensis*	*Aedes agypti, Anopheles stephensi*, *Culex pipiens* (Diptera: Cuclidae)

Table 4. Mosquitocidal activity of Cry and Cyt proteins (Modified after "Zeigler, 1999").

3.5 Resistance

One major problem with insects control via chemical insecticides is the evolution in insects of resistance to those insecticides. The use of *Bti* on biological control of mosquitoes has no resulted in the development of resistence in host populations. Laboratory attemps to induce resistance by continual exposure to *Bti* have generally failed to detect resistence (Lee & Chong 1985; Georghiou & Wirth 1997).

The lack of resistance development to *Bti* could be due to its complex mode of action, involving synergistic interactions between up to four proteins (Becker & Maragrit 1993). Use of a single protein from *Bti* for mosquito control resulted in resistance after only a few generations in the laboratory (Becker and Margalit 1993). Georghiou & Wirth (1997) also showed that resistance could be raised in only a few generations when single *Bti* toxin was used (i.e. Cry 4Aa, 4Ba, 10Aa or 11Aa), and was progressively more difficult to raise in mosquitoes with combinations of two, and three toxins. When all four *Bti* toxins were used, resistance incidence was remarkably low. On the other hand Wirth et al. (2005) have shown that the lack of resistance in *Bti* is due to the presence of the Cyt1Aa protein in the crystal. For example, *Culex quinquefascitus* populations resistant to CryIVA, Cry4B and Cry11A have been obtained in the laboratory but not mosquito larvae resistant to Cry and Cyt1Aa proteins (Georghiou & Wirth 1997)

3.6 Synergism

Bti produces four crystal proteins Cry (4Aa, 4Ba, 10Aa, and 11Aa) and two Cyt (1Aa and 2Ba) (Guerchicoff *et al.* 1997). No single crystal component is as toxic as the intact crystal complex (Chan *et al.* 1993; Wu *et al.* 1994; Chilcott & Ellar, 1998). One possible explanation for this is that two or more proteins act synergisticall, yielding a higher activity than would be expected on the basis of the specific toxicity of the individual protein (Finney, 1971). For example, the toxicity against mosquito larvae of Cyt1Aa is lower compared to each of the four Cry proteins (Crickmore et al. 1995). However, cytA can potentiate the activity of the toxins and synergistic interactions that seems to account for the high toxicity of the *Bti* strains (Delecluse et al. 1993).

Tabashnick (1992) proposed a method to measure synergistic effect. Using the proposed method discuss the data reported by two authors: in bioassays with *A. aegypti* larvae, Wu and Chang (1985) found that mixtures of the 27- and 65-kDa proteins from *B. thuringiensis* subsp. *israelensis* were more toxic than expected on the basis of their individual toxicities, however, Chilcott and Ellar (1988) concluded from their own data that no synergism between these two proteins occurred. With this new interpretation of Tabashnick method, both studies support the same conclusions: positive synergism between the 27-kDa protein (CytA) and either of the CryIV proteins (65 and 130 kDa) and no such synergism between CryIV proteins (65 and 130 kDa). Other studies have been carried out with the aim of increasing the synergistic activity of *Bti.* Ramirez-Suero et al. (2011) evaluated the synergistic effect of *S. griseus* and *Bt aizawai* chitinases with *Bt israelensis* spore-toxin complex against *Aedes aegypti* larvae. The synergistic factor values according to Tabashnik (1992) method were 2 and 1.4, respectively.

3.7 Effect of *Bti* on no-target organisms

Bti has no direct effect on aquatic organisms other than mosquitoes, blackflies and chironomids. Other aquatic organisms, such as shrimps, mites and oysters are generally unaffected (Glare & O'Callaghan, 1998). This large safety margin of preparations of *Bti* for

non-target organisms indicate their suitability for mosquito control programs in areas where protection of the natural ecosystem is important (Sinegre et al. 1980)
Several authors have reviewed the non target effects of *Bti* (Becker & Margalit 1993; Lacey & Mulla 1990). Field applications have often been monitored for effects on non-target organisms but no significant non-target effects have been reported (Ali, 1981; Jackson et al. 1994; Hershey et al. 1995)

4. Production of *Bti* by fermentation

4.1 Culture medium for *Bti* production

Commercial production of *Bti* is performed using culture media based on complex nutrients sources. The main purpose of the fermentation is to obtain high quantities of *Bti* crystals. The *Bti* parasporal crystal can account for up to 25% of the sporulated cell dry weight. To optimize the cry production it is necessary to have a suitable culture medium because the toxicity obtained at the end of the fermentation depend on the culture medium and operating conditions. The culture media that have been reported in the literature for high growth and sporulation can be used for any variety of *Bt*. Not always a high cell growth ensures an elevated Cry protein production or an increased insecticidal activity. Various culture mediums have been used for high growth and sporulation of *Bt* in the laboratory: 2XSG medium (Leighton & Doi, 1971), PA medium (Thorne, 1968), G-Tris medium (Aronson et al. 1971), CDGS medium (Nakata, 1964). Other media with inexpensive substrates have been reported by Pearson & Ward (1988), Smith (1982), Foda et al. (1985), Dulmage et al. (1970), Salama et al. (1983), Goldberg et al. (1980).

4.2 Factors affecting Cry production

There are several factors that influence the production of crystals: (1) Carbon source. Glucose is the most appropiate carbon source either for high *Bt* growth and sporulation (Smith, 1982). When glucose has been exhausted in the fermentation, the abscence of this can trigger sporulation. The use of one or other carbon source affects the biological activity and the morphology of the crystals (Dulmage, 1970). (2) Nitrogen source. An appropiate source of aminoacids provides high growth rates and high sporulation of *Bt* strains. Its absence delays sporulation and low yield in Cry proteins (Goldberg et al. 1980) (3) Carbon:Nitrogen ratio. Higher C:N rates glucose do not deplete at the end of fermentation and biomass yield decrease. Several authors have recomended a carbon nitrogen ratio of 7.5:1. (Salama et al. 1983; Foda et al. 1985) (4) Oxygen. High aeration rates are important for high spore and toxin formation. As k_La, increase biomass and Cry protein formation are increased (Rowe & Margaritis, 1987)(5) pH. Optimum pH for *Bt* growth is 6.8-7.2. If pH rises to 9.0 Cry protein can be dissolved (6) Temperature. Optimum temperature of *Bt* is 28-32°C. Higher temperatures favours plasmid losses or *Bt* mutants (Rowe & Margaritis, 1987).

5. Molecular biology of *Bti*

5.1 *Bti cry* and *cytA* genes

All *Bt* strains contain extrachromosomal DNA. *Bt* strains are well known for its numerous plasmids ranging in size from 1.5 MDa to 130 MDa. Plasmids have been found in each variety examined and the plasmid profiles appear to be strain specific. However these plasmid profiles depend on the media type and growth rate of the strain and can be readily

gained or lost (Federici, 1999). The mere presence of plasmids in *Bt* does not prove that they are involved in crystal formation, many non-crystalliferous bacteria also contain plasmids The *cry* genes are located on large plasmids although some Cry genes have been reported on the chromosome (Baume & Malvar, 1995). As mentioned earlier, *Bti* produces four different Cry proteins: CryIVA, CryIVB, Cry11A, and the cytolytic CytA protein (Hoffe and Whiteley, 1989). The Cry proteins are codified by *cryIVA*, c*ryIVB*, *cry11A*, and *cytA* genes, respectively. These genes responsible for the toxicity of *Bti* have been sequenced by various researchers (Table 5).

Gene name	GenBank Accesion No.	Coding Region	Reference
cryIVAa1	Y00423	1-3540	Ward &Ellar, 1987
cry4Aa2	D00248, E01676	393-3935	Sen *et al*. 1988
cry4Ba1	X07423, X05692	157-3564	Chungjatupornchai *et al*. 1988
cry4Ba2	X07082	151-3558	Tungpradubkul *et al*. 1988
cry4Ba3	M20242	526-3930	Yamamoto *et al.* 1988
cry4Ba4	D00247, E01905	461-3865	Sen *et al*. 1988
cry11Aa1	M31737	41-1969	Donovan *et al.* 1989
cry11Aa2	M22860	1-235	Adams *et al.* 1989
cyt1Aa1	X03182	140-886	Waalwijk *et al*. 1985
cyt1Aa2	X04338	509-1255	Ward & Ellar, 1986
cyt1Aa3	Y00135	36-782	Earp a& Ellar, 1987
cyt1Aa4	M35968	67-813	Galjart *et al*. 1987

Table 5. *cry* and *cytA* genes DNA sequences of *Bti.*

In *Bti* the elements responsible of the toxicity against mosquito larvae are located in a large plasmid of 72 MDa (125 kb) and contribute to the formation of a complex parasporal body (Aronson 1993). Figure 2 shows the partial map of the *Bti* 125 kb plasmid.

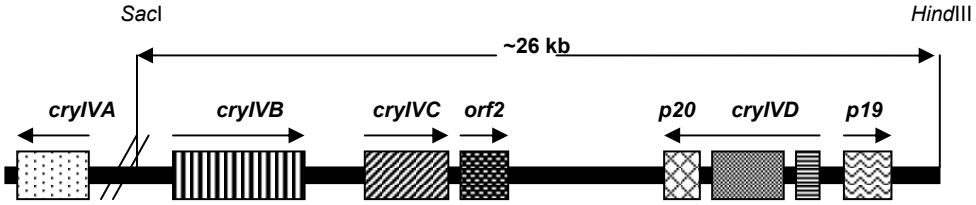

Fig. 2. Map of 26kb DNA fragment carrying the DNA genes responsibles of the toxicity of *Bti* (Modified after Ben-Dov *et al.* 1996).

5.2 *Bti* operon of *cryIVD* gene

The DNA sequence indicated that *cryIVD* gene is the second gene of an operon which includes three genes. A gene that encodes a 19 kDa polypeptide, *cryIVD* gene and a gene that encodes a 20 kDa polypeptide (Dervyn et al. 1995). Transcription of *cryIVD* gene in *Bti* is induced 9 h after the beginning of the sporulation. DNA sequence analysis and potential promoters are recognized by the RNA polimerase associated with the σ^{35} and σ^{28} , specific sigma subunit of the RNA polymerase genes related with control of sporulation of *B.*

thuringiensis. *cryIVA* and *cryIVB* promotors are activated in the mid-sporulation phase (Ben-Dov et al. 1996), *cryIVA* is regulated by the σ^{35} RNA polymerase gene and *cryIVB* is under control of σ^{35} (Yoshisue et al. 1994). *cytA* gene is transcribed by two promotors, pBtI and pBtII, regulated by the RNA polymerase σ^{35} and σ^{28}, respectively (Brown & Whiteley, 1988, 1990). These results have demostrated that *cryIVD* transcription is subjected to σ^{35} regulation.

5.3 Identication of *Bacillus thuringiensis* pesticidal crystal genes by PCR

The polymerase chain reaction (PCR) is a molecular tool widely used to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The identification of *Bt* toxin genes by PCR can partially predict the insecticidal activity of a given strain. Several studies have reported that the type of *cry* and *cyt* genes present in a *Bt* strain correlates to some extent with its insecticidal activity (Porcar & Juarez-Perez). Thus, the identification of the gene content in a *Bt* strain can be used to predict its insecticidal potential. The PCR-based identication of *B. thuringiensis* cry genes was first developed by Carozzi et al. (1991), who introduced this technique as a tool to predict insecticidal activity. They found correspondence with the toxicity predicted on the basis of the amplification product profiles. Carozzi et al. proposed PCR as an accurate, fast methodology for the identification of novel strains and the prediction of insecticidal activity of new isolates, and they also forecast the possible use of PCR for the discovery of previously unknown cry genes. highly conserved regions and recognizing entire cry gene subfamilies are often used in a preliminary screening prior to performing a second PCR with specific primers. The primers used to amplify *cry4A, cry4B, cry11A*, and *cytA* genes have been designed by various researchers and are shown in Table 6.

Direct	Sequences (5´⇒3)	Reverse Sequences (5´⇒3)	Amplifies/Product (bp)
EE-4A(d)	GGGTATGGCACTC AACCCCACTT*	Un4(r) GCGTGACATACCC ATTTCCAGGTCC*	cry4A/1529
Dip2A(d)	GGTGCTTCCTATTC TTTGGC**	Dip2B TGACCAGGTCCCT TGATTAC**	*cry4A*/1290
EE-4B(d)	GAGAACACACCTA ATCAACCAACT*	Un4(r) GCGTGACATACCC ATTTCCAGGTCC*	*cry4B*/1951
EE-11A(d)	CCGAACCTACTAT TGCGCCA*	EE-11A(r) CTCCCTGCTAGGA TTCCGTC*	*cry11A*/445
gral cyt1(d)	AACCCCTCAATCA ACAGCAAGG***	gral cyt(r) GGTACACAATACA TAACGCCACC***	*cyt1*/522-525

Table 6. PCR primers pairs and the cry or cyt genes they amplify of *Bti*. Sources: Ben-Dov *et al*. (1997)*, Carozzi et al. (1991)**, Bravo *et al*. (1998)***

5.4 Expression of *Bti* genes in other strains

Expression of *Bti* genes either individually or in combination in crystal-negative *Bt* or other strains have being carried on by several researchers. The genes encoding these proteins have been expressed in *Caulobacter* (Thanabalu et al. 1992), cyanobacteria (Manasherob et al. 2002; Murphy & Stevens 1992), *Escherichia coli* (McLean & Whiteley 1987; Tanapongpipat et al. 2003), *Bacillus subtilis* (Ward et al. 1986), and *Bt* (Crickmore et al. 1990). However, *Bt* toxins have been expressed as active or inactive toxins, especially when expressed in *E. coli* (Ogunjimi et al. 2002).

Quintano et al. (2005) reported the expression of *cry11A* from *Bti* in *S. cerevisiae*. The *cry11A* gene was expressed as fusion proteins with gluthathione *S*-transferase under the control of the *S. cerevisiae HXK1* promoter. The protein was purified by affinity chromatography using gluthathione *S*-transferase–Sepharose beads. Insecticidal activity against third-instar *Aedes aegypti* larvae of the recombinant *S. cerevisiae* cell extracts (LC_{50} = 4.10μg protein/mL) and purified GST-cry11A fusion protein (LC_{50} = 4.10μg protein/mL) was detected in cells grown in ethanol.

Servant et al. (1999) constructed a recombinant *B. sphaericus* strain containing the *cry11A* gene from *Bti*. They found an LC_{50} for the cry11A protein of 1.175 μg/mL against fourth-instar *A. aegypti* larvae. Poncet *et al.* (1997) constructed a recombinant *B. sphaericus* strain with *cry11A* and *p20* genes integrated into the chromosome. In this case, the LC_{50} value reported against third- instar *A. aegypti* larvae was 0.023 μg/mL. Xu *et al.* (2001) studied the expression of cry11A and *cry11A+p20* gene cluster in recombinant *E. coli* and *Pseudomonas putida*. They found that both recombinant bacteria contained higher levels of Cry11A protein when the adjacent *p20* gene was present on the same DNA fragment. Yamagiwa et al. (2004) reported that the solubilized cry11A protein, obtained from a nonrecombinant *Bti*, was less toxic against *Culex pipiens* larvae than the crystal itself (LC50 of 0.267 and 0.008 μg/mL, respectively). These authors obtained 2 GST fusion proteins of 36 and 32 kDa from cry11A. The LC50 against *C. pipiens* larvae obtained using both proteins were 0.818 μg/mL. In other study with the purpose to preserve the toxicity of sunlight-sensitive Cry proteins, Manasherob et al. (2002) constructed a transgenic cyanobacterium *Anabaena* PCC 7120 to express the genes *cry4Aa*, *cry11Aa* and an accesory protein (*p20*) under control of two tandem strong promotors. Cyanobacterium *Anabaena* can multiply in breeding sites of mosquito larvae and serve as their food source. Higher toxicity against *Aedes aegypti* larvae was obtained in this study.

6. Mode of action of *Bti* Cry proteins against mosquito larvae

6.1 Pore-forming-toxins

Bt Cry and Cyt toxins belong to a class of bacterial toxins known as pore-forming toxins (PFT) that are secreted as water-soluble proteins undergoing conformational changes in order to insert into, or to translocate across, cell membranes of their host (Bravo et al. 2007). In most cases, PFT are activated by host proteases after receptor binding inducing the formation of an oligomeric structure that is insertion competent. Finally membrane insertion is triggered, in most cases, by a decrease in pH that induces a molten globule state of the protein (Parker and Feil, 2005). Cry and Cyt proteins are PFT proteins. Both proteins are solubilized in the gut of suceptible diperans insects and proteolytic activated by midgut proteases. For the Cry 11Aa protoxin, proteolytic activation involves amino-terminal processing and intramolecular cleavage leading to two fragments of 36 and 32 kDa that remain associated and retain insect toxicity.

6.2 Mechanism of action of *Bti* toxins

An acepted model for Cry toxin action against mosquito larvae is that it is a multistage process. (i) Ingestion of Cry protein by the larvae (ii) Solubilization of the crystals in the alkaline midgut (iii) Proteolytic activation of the insecticidal solubilised protein (iv) Toxin binds to receptors located on the apical microvellus membrana of ephitelial midgut cell walls (v) Alter the toxin binds the receptor, it is though that there is a change in the toxin conformation allowing toxin insertion into the membrane (vi) Electrophysiological and biochemical evidence suggest that the toxins generate pores in the cell membrane, thus disturbing the osmotic balance, consecuently the cells swell and lyse (vii) The gut becomes paralyzed and the insect stops feeding. Most mosquito larvae die whitin few hours of ingestion, generally cease feeding within 1 hour, show reduced activity by two hours and die six hours after ingestion (Chicott et al. 1990; Marrone & Macintosh, 1993).

Several authors have studied the mechanisms of action of the δ-endotoxin of *Bti* on mosquito larvae. Thomas and Ellar (1983) found that δ-endotoxin active against mosquito larvae was inactivated by prior incubation with lipids extracted from ephitelial midgut *Aedes albopictus* cells. They reported that toxin binds to membrane lipids (phosphatidyl choline, sphingomyelin and phosphatidyl ethanolamine). According to their results, they proposed a mechanism in which the interaction of toxin with membrane lipids causes a detergent-like rearrangement of the lipids and as a consequence cytolisis. Others authors have corroborated these results: Cyt protein, unlike Cry toxins, do not recognize specific binding sites and do not bind to protein receptors, directly interact with membrane lipids inserting into the membrane and forming pores (Knowles *et al.* 1989; Promdonkoy & Ellar, 2000) or destroying the membrane by a detergent-like interaction (Butko, 2003).

The high efficacy of *Bti* is because of the production of multiple toxins with different modes of action. Perez *et al.* (2005) reported that Cyt1Aa protein functions as a receptor for the Cry11Aa toxin and suggest that this interaction explains the synergism between the Cyt1A and Cry11A proteins. Further, the Cyt proteins in *Bti* synergize the toxic effect of Cry11A and Cry4 toxins and, even more, suppresses the resistance to these Cry toxins (Wirth *et al.* 1997).

7. Formulation of *Bti* toxins

7.1 Potency in *Bti* formulations

Formulation is a preparation of an insecticide for a particular application method. Formulation plays an important rol in determining final virulence. The vast majority of the formulations of *Bt* have been developed to control agricultural and forest pests, mainly *Lepidoptera*. However, the feeding habits of *Lepidoptera* are different to feeding habits of *Diptera*. Mosquito larvae feed by filtering water and concentrate organic particles. Product formulations based on *Bti* should consider the mosquito larvae habits and the environmental conditions, promote that Cry proteins retain their toxic activity and promote that the larva have access to them. Products based on strains of *Bti* are given a potency based on bioassays on third or fourth instar mosquito larvae. Bioassays are conducted using 6 to 7 dilutions of the toxin by duplicate in 100 mL cups containing 20 third instar *Aedes aegypti* larvae. Duplicate cups with 20 mosquito larvae in 100 mL of deionized water without test material serves as a control (McLaughlin *et al.* 2004). Concentration-mortality data are obtained, transformed to a log-probit scale, and potency is obtained by comparing the estimated LC_{50} of a test substance with that of a standard with a known potency (de Barjac

1985). The international standard recognized for *Bti* is IPS82. LC_{50} and LC_{90} are the dose require to kill 50 and 90 percent of the mosquito larvae of a tested population after 24h tested duration. Each sample is bioassayed at least 3 times on various days and the results are average values. LC_{50} and LC_{90} are measured in micrograms or milligrams of material per liter, or parts per million (ppm).

For potency calculations, it is used the international recognized standard for mosquito assay, IPS82 (15 000 ITU/mg) provided by Institute Pasteur, Paris, France. Standard vials are kept at -18°C.

Product potency is calculated by the Abbott (1925) formula:

$$\text{Potency (A)} = \text{Potency (std)}\ LC_{50}\ (\text{std})/LC_{50}\ (\text{A})$$

where (a) is the product and (std) is the standard.

The size of the particle could be a factor that influences the potency of the toxin. A product with small particles is more homogenously distributed in the water than a product with larger particles and small particles which sink slower. Changes in LC_{50} are not necesary regarded to reflect changes in amount of toxin, but could be a function of particle size/distribution (Skovmand et al. 1997). Change of particle size also change LC_{50} . Decreasing particle increased LC_{50} and thus decrease the calculated potency. The slope measured between LC_{50} and LC_{90} values should have high value. Higher value will require a smaller quantity to kill a greater number of larvae. The slope of the dosage-mortality curve is in function of the heterogeneity of the product effect. If product availability is in function of particle sizes, particles with broad ranges of particle size distribution will also have low slopes.

7.2 *Bti* formulations

A variety of *Bti* formulations have been studied for mosquito control under laboratory and field conditions. *Bti* fluid formulations are not stable in heat and high humidity and cannot be stored for months under tropical conditions (McGuirre et al. 1996) . In many cases, and especially in areas exposed to the sun, the residual effect is very short (Leong et al. 1980) and the product has to be reapplied. Photoinactivation seems to be one of the major environment factors affecting the stability of *Bti* delta-endotoxin (Morris, 1983). Yu-Tien et al. (1993) reported that *Bti* completely lost its toxicity to mosquito larvae when exposed to irradiation at 253nm. Poszgay et al. (1987) showed that exposure of *B. thuringiensis* toxin to 40 h of ultraviolet light irradiation resulted in lost activity. Cry proteins inactivation by the solar radiation is the result of the destruction of the tryphtophan (Pusztai et al. 1991). Research and development efforts are focusing on formulations to avoid the ultraviolet light effect. Ramirez-Lepe et al. (2003) encapsulated *Bti* spore-toxin within aluminum/ carboxymethylcellulose using green malachite, congo red or ponceau red as photoprotective agents against ultraviolet light in lab conditions. The encapsulated form of the *Bti* spore-toxin complex with photoprotectors avoided the limitation in controlling mosquito larvae caused by ultraviolet light. Yu-Tien et al. (1993) achieved photoprotection of the spore-toxin complex by addition of melanin.

Other *Bti* formulations have been developed. For example, Ramirez-Suero et al. (2005) evaluated maltodextrin, nixtamalized corn flour and corn starch for entraping active materials in *Bti* spore-toxin complexes dried by aspersion. Dried products had water activity values below 0.7 suggesting that the formulations are long shelf-life because keep

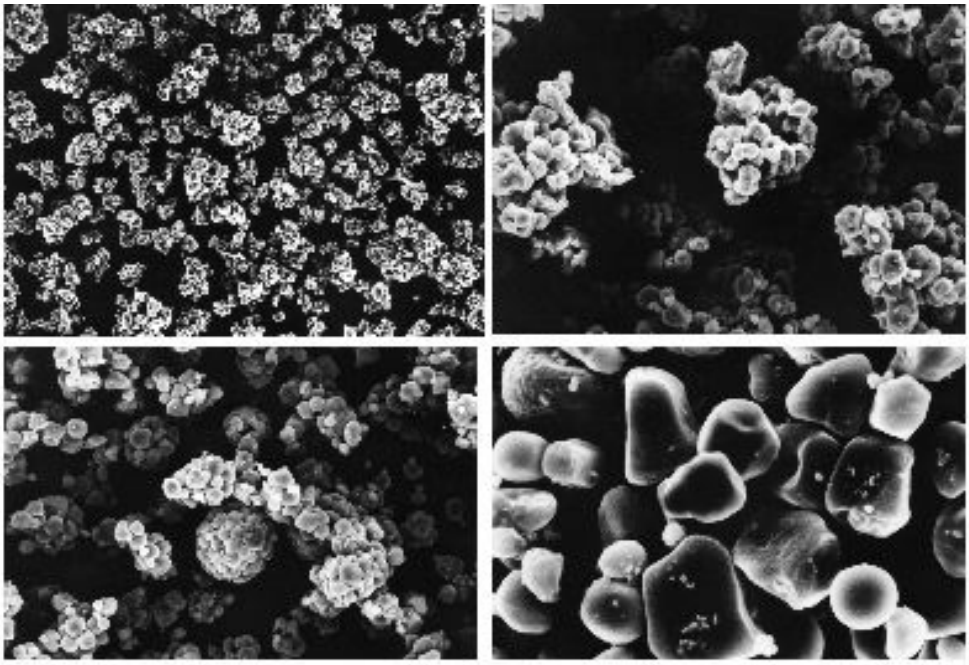

Fig. 3. Scanning electron micrograph of spray/dried formulation of *Bti* spore/toxin complex with Grits a) 379X and b)1500X and Nixtamalized corn flour c) 370X and d) 1500X.

the products without microorganisms for longer periods and increases the larval feeding and as a consequence have higher activity against mosquito larvae. Fig. 3 shows the scanning electron microscope of spray/dried formulations with grits and nixtamalized corn flour. It shows smoth spherical grits and corn particles entrapping the spore/toxin ingredient. Manasherob et al. (1996) encapsulated *Bti* in the protozoan *Tetrahymena pyriformis* and the activity against *A. stephensi* was enhanced 8 times when exposed to protozoan cells filled with *Bti* compared to exposed to the same concentration of *Bti* alone. Elcin (1995) encapsulated *Bti* in alginate microcapsules and increase its stability and its effect against *Culex* sp larvae. Another alternative to protect *Bti* crystals from ultraviolet light is obtaining mutants that protectrs *Bti* crystal. Hoti & Balaraman (1993) obtained a mutant of *Bti* that produced a brown pigment during sporulation, the pigment was identified as melanin. Other *Bti* formulations have been developed. Culigel superabsorbent polymer controlled-release system for the slow release of *Bti* to mosquito larvae (Levy et al. 1990). Combination of chemical and biological agents susch as insect growth regulator s-methoprene + *Bti* (Bassi et al. 1989). Sprayed-dried *Bti* powder as a fizzy tablet (Skovmand & Eriksen 1993). Floating bait formulations designed to improve the effect of bacterial toxins, especially against *Anopheles* spp (Aly et al. 1987).

Highly concentrated liquid formulations are available for control of floodwater mosquitoes while formulations which float for as long possible have been developed for use in fast-flowing or turbulent waters. Formulations which settle and persist at the bottom are required for buttom feeders. Granules which float on the surface are the most effective against surface feeders such as *Anopheles* spp. (Mulla et al. 2004).

Briquettes for mosquitoes with continual successive generations like *Culex* spp (Becker & Margalit 1993; Kase & Branton 1986). Briquetts or pellets, in particular seem to be useful for overcoming lack of persistence, which is one of main limitations of *Bti*. Granules using plant, such corn (maiz) grits or clay carriers are particular useful in aerial application to breeding sites with dense foliage as salt marshes or rice fields. Sustained release formulations such as floating briquettes or semi submersible pellets are designed to provide long-lasting larvicidal activity in containers or small ponds. Ingestion of the toxin depends on the rate of feeding, the rate at which the toxin falls to the bottom of the pool and becomes inaccesible, and competition to ingestion from other suspended organic materials. In turbid and polluted waters the rate of application needs to be at least two-fold greater than in clear water (Mulla et al. 1985). The feeding habits of mosquito larvae influence formulation design. *Anopheles* larvae are surface feeders and ingest particulate material from water surface such as yeast or flour and filter feed poorly. This has led to the development of formulations that present the toxin at, or just below, the water surface and such prepartions are particulary effective against certain *Anopheles* larvae (Aly et al. 1987)

8. Field application

One of the major drawbacks in the use of *Bti* is its rapid inactivation (24-48 h) in the environment (Mulla et al. 1993). Thus larvae populations of stagnant water mosquitoes recover within 5-7 days following treatment. Therefore the use of *Bti* is limited by the low efficacies of current preparations under field conditions (Tyanum & Mulla 1999). Since there is a little persistence of the toxin further applications are necessary to effect continuous control. Formulation and applications techniques can extend the persistence of activity for over one month in some situations, but activity remains sensitive to factors like UV degradation. In areas exposed to the sun, the residual effect is very short (Leong et al. 1980) and the product has to be reapplied. Other factors that affect the toxicity of *Bti* are particle sedimentation (Rushed & Mulla, 1989), protein adsorption onto silt particles, organic matter, elevated temperatures (Ohana et al. 1987), consumption by other organisms to which the toxin is not lethal (Blaustein and Margalit, 1991), dissolved tannins (Lord & Undeen, 1990) and inactivation by sunlight. Other factors that have been observed are that *Bti* does not recycle, under simulated field conditions, is unable to germinate and multiply in mud at the botton of pools although it did remain viable viable for up to 22 days, and higher water depth where applied decrease its activity (Ohana et al. 1987)

Early reports showed that a primary powder formulations of *Bti* had virtually no residual effect against mosquito larvae beyond application (Aguilar-Meza et al. 2010), although the delta-endotoxin remained chemically stable in neutral and acid waters (Sinegre et al 1980). Extend persistence with *Bti* is possible through use of improved formulations. Gunasekaran *et al.* (2002) tested a floating sustained release formulation of *Bti* in polluted water habitats against *Culex quinquefasciatus* larvae. Briquetts may result in more prolonged control than liquid formulations as these products have greater persistence through slow release (Kase & Branton 1986).

On the other hand, Aguilar-Meza et al. (2010) tested the residual insecticidal activity after field exposure of an aluminum-carboxymethylcellulose microencapsulated formulation of *Bti* spore-toxin complex with malachite green as photoprotective agent. The formulation improved the activity against *Aedes aegypti* larvae for 30 days and was comparable to that of the chemical insecticide temephos.

9. Conclusions

Bti is a bacterium that has been applied with success in biological control programs against mosquitoes and flies larvae all over the world. The study in each of its facets addressed in this review will open new perspectives to improve their effectiveness in biological control.

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11. References

Abbott, W.S. (1925). A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology*, Vol. 18, pp. 265-267.

Adams, L.F.; Visick, J.E. & Whiteley H.R. (1989). A 20-kilodalton protein is required for efficient production of the *Bacillus thuringiensis* subsp. *israelensis* 27-kilodalton crystal protein in *Escherichia coli*. *Journal of Bacteriology*, Vol 171, pp. 521-530

Aguilar-Meza, O.; Ramırez-Suero, M.; Bernal, J.S. & Ramırez-Lepe, M. (2010). Field evaluation against *Aedes aegypti* larvae of aluminum-carboxymethylcellulose-encapsulated formulation of *Bacillus thuringiensis* serovar *israelensis*. *Journal of Economic Entomology*, Vol.103, pp. 570–576

Aronson, A.I. Angelo, N. & Holt, S.C. (1971). Organic Nutrients Required For Growth and Sporulation of *Bacillus Cereus*. *Journal of Bacteriology*, Vol.106, p.p.1016-1025.

Ali, A. (1981). *Bacillus thuringiensis* serovar. *israelensis* (ABG-6108) against chironomid midges and some nontarget invertebrates. *Journal of Invertebrate Pathology* Vol 38, pp. 264–272.

Aly, C.; Mulla, M.S. & Schnetter, W. (1987). Floating formulations increase activity of *Bacillus thuringiensis* var. *israelensis* against *Anopheles* larvae. *Journal of the American Mosquito Control Association* Vol 3, pp. 583–588.

Aly, C.; Mulla, M.S.; Xu, B.Z. & Schnetter, W. (1988). Rate of ingestion by mosquito larvae (Diptera:*Culicidae*) as a factor in the effectiveness of a bacterial stomach toxin. *Journal of Medical Entomology,* Vol 25, pp.191–196.

Aoki, K & Chigasaki, Y. (1915) Uber des toxin von sog. Sotto-bacillen Mitt. Med. Fak. Kais. Univ. Tokyo. Vol. 14, pp. 59-80

Aronson, A.I.; Han, E.S; McGaughey, W & Johnson, D. (1991). The solubility of inclusion proteins from *Bacillus thuringiensis* is dependent upon protoxin composition and is a factor in toxicity to insects. *Applied and Environmental Microbiology*, Vol 57, pp.981-986

Ash, C. & Collins, M.D. (1992). Comparative analysis of 23s ribosomal RNA gene sequences of *Bacillus anthracis* and emetic *Bacillus cereus* determined by PCR-direct sequencing. *FEMS Microbiology Letters,* Vol 73, pp. 75-80

Bassi, D.G.; Weathersbee, A.A.; Meisch, M.V. & Inman, A. (1989). Efficacy of Duplex and Vectobac against *Psorophora columbiae* and *Anopheles quadrimaculatus* larvae in Arkansas ricefields. *Journal of the American Mosquito Control Association,* Vol 5, pp. 264-266.

Bauer, L.S. (1995). A threat of the insecticidal crystal protein of *Bacillus thuringiensis*. *Florida Entomologist*, Vol 78, pp. 414-443

Baum, J. A. & T. Malvar, T. (1995). Regulation of insecticidal crystal protein production in *Bacillus thuringiensis*. *Molecular Microbiology,* Vol 18, pp. 1-12.

Bartlet, B.R. (1964). Integration of Chemical and Biological Control. In: *Biological Control of insect pests and weeds*, P. DeBach, (Ed.), 489-511, Chapman & Hall, London.

Becker, N. (2000). Bacterial control of vector-mosquitoes and black flies. In *Entomopathogenic Bacteria: From Laboratory to Field Application*, (Ed.) J.F. Charles, A. Delécluse and C. Nielsen-LaRoux), pp. 383-398. Dordrecht, The Netherlands: Kluwer.

Becker, N. & Margalit, J. (1993). Use of *Bacillus thuringiensis israelensis* against mosquitoes and blackflies. In *Bacillus thuringiensis, an EnvironmentalBiopesticide: Theory and Practice,* P. F. Entwistle, J. S. Cory, M. J. Bailey and S. Higgs (Ed.)), pp. 147-170. John Wiley & Sons, New York.

Ben-Dov, E.; Zaritsky, A.; Dahan, E.; Barak, Z.; Sinai, R.; Manasherob, R.; Khamraev, A.; Troitskaya, E.; Dubitsky, A.; Berezina, N. & Margalith, Y. (1997). Extended screening by PCR for seven *cry*-group genes from field-collected strains of *Bacillus thuringiensis*. *Applied and Environmental Microbiology*, Vol 63, pp. 4883-4890

Bravo, A.; Sarabia, S.; Lopez, L.; Oniveros, H.; Abarca, C.; Ortiz, A.; Ortiz, M.; Lina, L.; Villalobos F.J.; Peña, G.; Nuñez-Valdez, M.E.; Soberón, M. & Quintero. R. (1998). Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain collection. *Applied and Environmental Microbiology,* Vol 64, pp. 4965-4972

Bravo, A.; Gill, S.S. & Soberon, M. (2007). Mode of Action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon,* Vol.49, No.4 (March 2007). pp.423-435

Brown, K. L. & Whiteley, H.R. (1988). Isolation of a *Bacillus thuringiensis* RNA polymerase capable of transcribing crystal protein genes. *Proceedings of the National Academy of Sciences,* Vol 85, pp. 4166–4170.

Brown, K. L., & H. R. Whiteley. (1990). Isolation of the second *Bacillus thuringiensis* RNA polymerase that transcribes from a crystal protein gene promoter. *Journal of Bacteriology,* Vol 172, pp. 6682–6688.

Butko, P. (2003). Cytolytic toxin Cyt11A and its Mechanism of Membrane Damage: Data and Hypotesis. *Applied and Environmental Microbiology*. Vol69, No.5, (May 2003) pp 2415-2422

Carozzi, N. B.; Kramer, V.C.; Warren, G.W.; Evola, S. & Koziel, M.G. (1991). Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. *Applied and Environmental Microbiology,* Vol 57, pp. 3057-3061

Crickmore, N.; Zeigler, D.R.; Feitelson, J.; Schnepf, E.; Vanrie, J.; Lereclus, D.; Baum, J. & Dean, D.H. (1998). Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiology and Molecular Biology Reviews,* Vol 62, pp. 807-813

Crickmore, N.; Nicholls, C.; Earp, D.J.; Hodgman, T.C.; & Ellar, D.J. (1990). The construction of *Bacillus thuringiensis* strains expressing novel entomocidal ™-endotoxin combinations. *Journal of Biochemistry,* Vol.270, pp. 133–136.

Chang, C.; Dai, S.M Frutos, R.; Federici, B.A. & Gill, S.S. (1992). Properties of a 72-kilodalton mosquitocidal protein from *Bacillus thuringiensis* subsp. morrisoni PG-14 expressed in *Bacillus thuringiensis* subsp. *kurstaki* by using the shuttle vector pHT3101. *Applied and Environmental Microbiology,* Vol 58, pp. 507-512

Charles, J. F. & deBarjac, H. (1982). Sporulation et crystal- logenese de *Bacillus thuringiensis* var *israelensis* en microscopie electronique.*Annals of Microbiology* (Inst.Pasteur)133A:425-442.

Chen, M.L.& Tsen H.Y. (2002). Discrimination of Bacillus cereus and Bacillus thuringiensis with 16S rRNA and gyrB gene based PCR primers and sequencing of their annealing sites. *Journal of Applied Microbiology*. Vol.92, pp. 912–919

Chilcott, C. N. & Ellar, D.J. (1988). Comparative toxicity of *Bacillus thuringiensis* var. *israelensis* crystal proteins *in vivo* and *in vitro. Journal of General Microbiology,* Vol.134, pp.2552-2558

Chilcott, C.N.; Knowles, B.H.; Ellar, D.J. & Drobniewski, F.A. (1990). Mechanism of Action of *Bacillus thuringiensis israelensis* Parasporal Body. In *Bacterial Control of Mosquitoes and Blackfles,* eds de Barjac, H. & Sutherland, D.J. pp. 45-65. New Brunswick : Rutgers University Press.

Chungjatupornchai, W.; Höfte, H.; Seurinck, J.; Angsuthanasombat, C. & Vaeck, M. (1988). Common features of *Bacillus thuringiensis* toxins specific for *Diptera* and *Lepidoptera*. *European Journal of Biochemistry*, Vol.173, pp.9-16

de Barjac, H. & Bonnefoi, A. (1973). Mise au point sur la classification des *Bacillus thuringiensis. Entomophaga.* Vol 18, pp. 5-17

de Barjac, H. 1978. A new subspecies of *Bacillus thuringiensis* very toxic for mosquitoes; *Bacillus thuringiensis* serotype H-14. *Compte Rendu Académie Sciences Paris Series D*. Vol. 286 pp.797-800.

de Barjac, H. & Frachon, E. (1990). Classification of *Bacillus thuringiensis* Strains Entomophaga. Vol. 35, pp. 233-240.

de Barjac, H. & Larget-Thiery, I. (1985). Characteristics of IPS82 standard for bioassay of *B. thuringiensis* H-14 preparations. WHO Rep/VBC/84.892.

Dervyn, E.; Poncet, S.; Klier, A. & Rapoport, G. (1995). Transcriptional regulation of the *cryIVD* gene operon from *Bacillus thuringiensis* subsp. *israelensis*. *Journal of Bacteriology,* Vol.177, pp.2283-2291

Donovan, W. P.; Dankocsik, C. & Gilbert, M.P. (1988). Molecular characterization of a gene encoding a 72-kilodalton mosquito-toxic crystal protein from *Bacillus thuringiensis* subsp. *israelensis*. *Journal of Bacteriology,* Vol.170, pp.4732-4738

Dulmage, H.T. (1970). Production of the spore- endotoxin complex by variants of *Bacillus thuringiensis* in two fermentation media. *Journal of Invertebrate Pathology*, Vol.16, pp.385-389.

Earp, D.J. & Ellar, D.J. (1987). *Bacillus thuringiensis* var. *morrisoni* strain PG14: nucleotide sequence of a gene encoding a 27 kDa crystal protein. *Nucleic Acids Research*, Vol 15, pp. 3619-3624

Dervyn, E.; Poncet, S.; Klier, A,; Rapoport, A.; Yoshisue, H.; Nishimoto, T.; Sakai, H. & Komano, T. (1994). Identification of a promoter for the crystal protein-encoding gene *cryIVB* from *Bacillus thuringiensis* subsp. *israelensis*. *Gene* Vol.137, pp.247–251

Debro, L.; Fitz-James, P.C. &Aronson, A. (1986). Two different parasporal inclusions are produced by *Bacillus thuringiensis subsp. finitimus. Journal of Bacteriology,* Vol.58, pp. 507-512

Delafield, F.P.; Somerville, H.J. & Rittenberg S.C. (1968). Immunological homology between crystal and spore protein of *Bacillus thuringiensis. Journal of Bacteriology,* Vol.96 pp.713-720

Dunkle, R.L., & B.S. Shasha. 1989. Response of starch encapsulated *Bacillus thuringiensis* containing ultraviolet screens to sunlight. Environ. Entomol. 18: 1035-1041.

Drobniewski, F.A. (1993) Onchocerciasis and the Onchocerciasis Control Programme. *Microbiology Europe* Vol.1, pp. 26-28,

Elcin, Y.M.; Cokmus, C. & Sacilik, C.S. (1995). Aluminum carboximethylcellulose encapsulation of *Bacillus sphaericus* 2362 for control of *Culex* spp. (Diptera: *Culicidae*) larvae. *Journal of Economic Entomology* Vol.88 pp. 830-834

Federici, B.A. (1999). *Bacillus thuringiensis* in Biological Control. . In: *Handbook of Biological Control.* T. Fisher (Ed.)Academic Press (Ed.) 575-593, ISBN 10: 0-12-257305-6

Federici, B.A.; Park, H.W.; Bideshi, D.K.; Wirth M.C. & Johnson, J.J. (2003). Review. Recombinant Bacteria for Mosquito Control. *The Journal of Experimental Biology*, Vol.206, pp.3877-3885

Finney, D.J. (1971). Probit Analysis. Cambridge Univ. Press, Cambridge, United Kingdom.

Foda M.S.; Salama, H.S. & Selim, M. (1985). Factors afecting growth physiology of *Bacillus thuringiensis. Applied Microbiology and Biotechnology*, Vol.22, pp.50-52.

Galjart, N. J.; Sivasubramanian, N. & Federici, B.A. (1987). Plasmid location, cloning and sequence analysis of the gene encoding a 23-kilodalton cytolytic protein from *Bacillus thuringiensis* subsp. *morrisoni* (PG-14). Current Microbiology, Vol.16, pp. 171-177

Glare T.R. & O'Callaghan M. (1998). Environmental and Health Impacts of *Bacillus thuringiensis israelensis. Report for the Ministry of Health.* Biocontrol & Biodiversity, Grasslands Division, Ag. Research. Lincoln, New Zealand.

Goldberg, L. J. & Margalit, J. (1977). A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii, Uranotaenia unguiculata, Culex univitattus, Aedes aegypti* and *Culex univitattus*. *Mosquto News,* Vol.37, pp. 355–358

Georghiou, G.P. & Wirth, M.C. (1997). Influence of Exposure to Single versus Multiple Toxins of *Bacillus thuringiensis* subsp. *israelensis* on Development of Resistance in the Mosquito *Culex quinquefasciatus* (Diptera: *Culicidae*). *Applied and Environmental Microbiology,* Vol.63, No. 3, pp.1095-1101

Goldberg, I.; Sneh, B.; Battae, E. & Klein, D. (1980). Opt i mi zat i on o f a medium for a high product i on o f spore-crystal preparat i on o f *Bacillus thuringiensis* effective against Egyptian cotton leaf worm *Spodoptera littorallis. Biotechnology Letters* Vol.2, pp. 419-426.

Hannay, L.C. (1953). Cristalline inclusion in aerobic spore forming bacteria. Nature. Vol. 172, pp. 1004.

Hannay, C.L. & Fitz-James P.C. (1955). The protein crystals of *Bacillus thuringiensis Berl. Canadian Journal of Microbiology.* Vol 1, pp. 694-710.

Hershey, A. E., Shannon, L., Axler, R., Ernst, C. & Mickelson, P. (1995). Effects of methoprene and *Bti* (*Bacillus thuringiensis* var. *israelensis*) on non-target insects. *Hydrobiologia,* Vol. 308, pp.219-227

Höfte, H. & Whiteley, H.R. (1989). Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiology Reviews* Vol.53 pp. 242-255.

Hoti, S.L, & Balaraman, K. (1993). Formation of melanin-pigment by a mutant of *Bacillus thuringiensis* H-14. *Journal of General Microbiology* Vol.139, pp. 2365-2369.

Husz, B. (1931). Experiments during 1931 on the use of *Bacillus thuringiensis Berl*. in controlling the corn borer. *Science Reports International of Corn Borer Investigation*, Vol. 4, pp. 22-23.

Ibarra, J. E. & Federici, B. A. (1986). Isolation of a relatively nontoxic 65-kilodalton protein inclusion from the parasporal body of *Bacillus thuringiensis*subsp. *israelensis*. *Journal of Bacteriology,* Vol.165, pp.527-531

Ignofo, C.M., Shasha, B.S. & Shapiro, M (1991). Sunlight ultraviolet protection of the *Heliothis* nuclear polyhedrosis virus through starch encapsulation technology. *Journal of Invertebrate Pathology,* Vol 57: 134-136.

Ishiwata, S. (1901). On a kind of severe flacherie (sotto disease). *Dainihon Sanshi Kaiho* Vol.114, pp.1-5

Iwabushi, H. (1908) Popular silkworm Pathology A.A. Meibundo (Ed.) Tokyo, pp. 428.

Kase, L.E. & Branton, L. (1986). Floating particle for improved control of aquatic insects. U.S. Pat. 4631857

Knowles, B. H.; Blatt, M.R.; Tester, M.; Horsnell, J.M.; Carroll, J.; Menestrina G. & Ellar, D.J. (1989). A cytolytic delta-endotoxin from *Bacillus thuringiensis* var. *israelensis* forms cation-selective channels in planar lipid bilayers. *FEBS Letters*, Vol.244, pp.259–262

Krieg V.A.; Huger A.M.; Langenbruch G.A. & Schnetter W. (1983). *Bacillus thuringiensis* var. *tenebrionis*: Ein neuer, gegenüber lair ven von coleopteren wirksamer pathotyp. *Z. Äng. Ent.* Vol.96, pp. 500-508

Jacobs, S.E. (1950).Bacteriological control of the flour moth *Ephestia kuhniella*. *Proceedings of the Society of Apply Bacteriology,* Vol.13, pp. 83-91.

Jackson, J. K.; Sweeney, B. W.; Bott, T. L.; Newbold, J. D. & Kaplan, L. A. (1994). Transport of *Bacillus thuringiensis* var. *israelensis* and its effect on drift and benthic densities of nontarget macroinvertebrates in the Susquehanna River, northern Pennsylvania. *Canadian Journal of Fisheries and Aquatic Sciences* Vol.51, pp.295-314

Johnson, M.W. & Tabashnik B.E. (1999). Enhanced Biolgical Control Through Pesticide Selectivity. In: *Handbook of Biological Control.* T. Fisher (Ed.)Academic Press (Ed.) 297-317, ISBN 10: 0-12-257305-6

Lacey L.A.; Urbina, M.J. & Heitzzman, C.M.(1989). Sustained release formulations of *Bacillus sphaericus* and *Bacillus thuringiensis* (H-14) for control of container-breeding *Culex quinquefasciatus*. *Mosquito News* Vol.44, pp.26-32.

Lacey, L. A. & Mulla, M. S. (1990). Safety of *Bacillus thuringiensis* var. *israelensis* and *Bacillus sphaericus* to nontarget organisms in the aquatic environment. In *Safety of Microbial Insecticides.* Laird-M, Lacey-LA and Davidson-EWSO (Ed.), pp. 259 pp. CRC Press Inc.; Boca Raton; USA.

Leighton, T.J. & Doi, R.H.(1971). The Stability of Messenger Kibonucleic Acid during Sporulation in *Bacillus subtilis*. *The Journal of Biological Chemistry*. Vol.246, pp.3189-3195.

Leong, K.L.; Cano, R.J. & Kubinski, A.M (1980). Factors affecting *Bacillus thuringiensis* total field persistence. *Environmental Entomology*, Vol.9 pp.593-599.

Levy, R.; Nichols, M.A. & Miller, T. W. (1990). Culigel superabsorbent polymer controlled-release system: application to mosquito larvicidal bacilli. *Proceedings and Abstracts, Vth International Colloquium on Invertebrate Pathology and Microbial Control, Adelaide, Australia* , 107

Lord, J.C. & Undeen A.H. (1990). Inhibition of the *Bacillus thuringiensis* var. *israelensis* toxin by dissolved tannins. Environmental Entomology, Vol.19 pp.1547-1551

Manasherob, R., Ben-Dov, E., Margalit, J., Zaritsky, A. & Barak, Z. (1996). Raising activity of *Bacillus thuringiensis* var. *israelensis* against *Anopheles stephensi* larvae by encapsulation in *Tetrahymena pyriformis* (Hymenostomatidia: Tetrahymenidae). *Journal of the American Mosquito Control Association,* Vol.12, pp. 627-631

Manasherob, R.; Ben-Dov, E.; Xiaoqiang, W.; Boussiba, S., & Zaritsky, A. (2002). Protection from UV-B damage of mosquito larvicidal toxins from *Bacillus thuringiensis* subsp. *israelensis* expressed in *Anabaena* PCC 7120. *Current Microbiology,* Vol 45, pp. 217–220.

Marrone, P.G. & MacIntosh, S.C. (1993). Resistance to *Bacillus thuringiensis* and Resistance Management. *In: Bacillus thuringiensis*, an Environmental Biopesticide: Theory and

Practice, Entwistle, P. F., Cory, J. S., Bailey, M. J., and Higgs, S., Eds., John Wiley and Sons, Chichester, UK, pp.221-235.

McDonald B.A. & Linde C. (2002). Pathogen Population Genetics, Evolutionary Potential, and Durable Resistance. *Annual Review of Phytopathology*, Vol. 40, pp. 349-379

McGuirre, M.R.; Shasha, B.S.; Eastman, C.E. & Sagedchi H.O. (1996). Starch and fluor-based sprayable formulations: Effect on rainfastness and solar stability of *Bacillus thuringiensis*. *Journal of Economic Entomology*, Vol.89, pp. 863-869

McLaughlin, R.E.; Dulmage, H.T.; Alls, R.; Couch, T.L.; Dame, D.A.; Hall, I.M.; Rose, R.I. &.Versol, P.L. (1984). U.S. standard bioassay for the potency assessment of *Bacillus thuringiensis* var. *israelensis* serotype H-14 against mosquito larvae. *Bulletin of the Entomological Society of America*, Vol.30, pp. 26-29

McLean, K.M. & Whiteley, H.R. (1987). Expression in *Escherichia coli* of a cloned crystal protein gene of *Bacillus thuringiensis* subsp. *israelensis*. *Journal of Bacteriology,* Vol.169, pp. 1017–1023

Mattes, O. (1927) Parasitare kronkheiten der mehlmotten larven und versuche uber ihre Verwendborkeit als biologische Bekampfungsmittel. *Sisher Ges. Beforder. Ges Naturw. Marburg*. Vol.62., pp. 381-417

Metalnikov, S.; Hengula, B. & Strail, D.M. (1930). Experiments on the application of bacteria against the corn borer. *Science Reports International Corn Borer Investigation,* Vol3, pp. 148-151.

Mikkola, A. R.; Carlberg, G.A.; Vaara, T. & Gyllenberg, H.G. (1982). Comparison of inclusions in different *Bacillus thuringiensis* strains. An electron microscope study. FEMS Microbiology Letters, Vol.13, pp.401-408

Mitani, K & Watari, J. (1916). A new method to isolate the toxin of *Bacillus sotto* Ishiwata by passing through a bacerial filter and a preliminary report on the toxic action of this toxin to the silkworm larvae. *Archi Gensanshu Serzojo Hokoku.* Vol 3, pp. 33-42.

Metcalf, R.L. (1986). The Ecology of Insecticides and the Chemical Control of Insects. *In Ecological Theory and Integrated Pest Managment in Practice*, M. Kogan (Ed.) 251 297 John Wiley & Sons, New York.

Morris, O.N. (1983). Protection of *Bacillus thuringiensis* from inactivation from sunlight. *Canadian Entomology*, Vol 115, pp.1215-1227.

Mulla, M. S. & Darwazeh, H.A. (1985). Efficacy of formulations of *Bacillus thuringiensis* H-14 against mosquito larvae. *Bulletin fo the Society of Vector Ecology.* Vol.10, pp.14–19

Mulla, M. S.; Darwazeh, H.A.; Ede, L.; Kennedy, B. & Dulmage H.T. (1985). Efficacy and field evaluation of *Bacillus thuringiensis* (H-14) and *B. sphaericus* against floodwater mosquitoes in California. *Journal of American Mosquito Control Association* Vol1, pp.310–315.

Mulla, M. S.; Darwazeh, H.A. & Zgomba, M. (1990). Effect of some environmental factors on the efficacy of *Bacillus sphaericus* 2362 and *Bacillus thuringiensis* (H-14) against mosquitoes. *Bulletin of the Society of Vector Ecology,* Vol.15, pp.166–175.

Mulla, M. S.; Chaney, J.D. & Rodchareon, J. (1993). Elevated dosages of *Bacillus thuringiensis* var. *israelensis* fail to extend control of *Culex* larvae. *Bulletin of the Society of Vector Ecology*, Vol.18, pp.125–132.

Mulla, M. S.; Thavara, U.; Tawatsin, A. & Chompoosri, J. (2004). Procedures for the evaluation of field efficacy of slow-release formulations of larvicides against *Aedes aegypti* in water-storage containers. *Journal of the American Mosquito Control Association* Vol.20, pp.64–73

Nakata, H.M. (1964). Organic Nutrients Required For Growth And Sporulation Of *Bacillus Cereus*, *Journal of Bacteriology,* Vol.88, pp.1522-1524;

Ohana, B.; Margalit, J. & Barak, Z. (1987). Fate of *Bacillus thuringiensis* subsp *israelensis* under simulated field conditions. *Applied and Environmental Microbiology,* Vol.53, pp.828-831.

Ogunjimi, A.A.; Chandler, J.M.; Gbenle, G.O.; Olukoya, D.K., & Akinrimisi, E.O. (2002). Heterologous expresión of *cry2* gene from a local strain of *Bacillus thuringiensis* isolated in Nigeria. *Biotechnology Applied Biochemistry*, Vol.36, pp.241–246

Pearson, D. & Ward, O.P. (1988). Effect of culture conditions on growth and sporulation of *Bacillus thuringiensis* susp. *israelensis* and development media for production of the protein crystal endotoxin. *Biotechnology Letters*, Vol.10, pp.451-456.

Perez, C.; Fernandez, L.E.; Sun, J.; Folch, J.L.; Gill, S.S. & Soberon M. (2005*). Bacillus thuringiensis* subsp. *israelensis* Cyt1Aa synergizes Cry11Aa Toxin by Functioning as a Membrane-Bound Receptor. *Proceedings of the National Academy of Sciences*. Vol 102, No. 51. pp. 18303-18308

Poncet, S.; Bernard, C.; Dervyn, E.; Cayley, J.; Klier, A.; & Raport, G. (1997). Improvement of *Bacillus sphaericus* toxicity against dipteran larvae by integration, via homologous recombination, of the *cry11A* toxin gene from *Bacillus thuringiensis* subsp. *israelensis*. *Applied and Environmental Microbiology,* Vol.63, pp 4413–4420

Porcar, M. & Juarez-Perez, V. (2003). PCR-based identification of *Bacillus thuringiensis* pesticidal crystal genes. *FEMS Microbiology Reviews*. Vol. 26, pp. 419-432.

Pozsgay, M.; Fast. P.; Kaplan, H. & Carey, P.R. (1987). The effect of sunlight on the protein crystals from *Bacillus thuringiensis* var. *kurstaki* HD1 and NRD12: A raman spectroscopy study. *Journal of Invertebrate Pathology,* Vol.50 pp.620-622

Priest, F.G. (1992). A review. Biological control of mosquitoes and other biting flies by *Bacillus sphaericus* and *Bacillus thuringiensis*. *Journal of Applied Bacteriology,* Vol.72, pp.357-369.

Promdonkoy, B. & Ellar, D.J. (2000). Membrane pore architecture of a cytolytic toxin from *Bacillus thuringiensis. Journal of Biochemistry.*Vol. 350, pp.275–282.

Pusztai, M.; Fast, P.; Gringorten, L., Kaplan, H.; Lessard, T. & and Carey, P.R. (1991). The mechanism of sunlight-mediated inactivation of *Bacillus thuringiensis* crystals. *Journal of Biochemistry*, Vol.273, pp.43-47

Quintana-Castro, R.; Ramírez-Suero, M.; Moreno-Sanz, F. & Ramírez-Lepe, M. (2005). Expression of the *cry11A* gene of *Bacillus thuringiensis* ssp. *israelensis* in *Saccharomyces cerevisiae,.Canadian Journal of Microbiology, Vol.*51, pp.165-170

Ramırez-Lepe, M.; Aguilar, O.; Ramırez-Suero, M. & Escudero, B. (2003). Protection of the spore-toxin complex of *Bacillus thuringiensis* serovar *israelensis* from ultraviolet irradiation with aluminum-CMC encapsulation and photoprotectors. *Southwestern. Entomology,* Vol.28, pp.137-143.

Ramírez-Suero, M.; Robles-Olvera, V. & Ramírez-Lepe, M. (2005). Spray-dried *Bacillus thuringiensis* serovar *israelensis* formulations for control of *Aedes aegypti* larvae. *Journal of Economic Entomology*, Vol.98, pp.1494-1498

Ramırez-Suero, M.; Valerio-Alfaro, G.; Bernal, J.S. & Ramirez-Lepe M. (2011). Synergisitic effect of chitinases and *Bacillus thuringiensis israelensis* spore-toxin complex against *Aedes aegypti* larvae. *The Canadian Entomologist* Vol,143: pp. 157–164.

Rawlins, S.C. (1989) Biological control of insect pests affecting man and animals in the tropics. *CRC Critical Reviews in Microbiology* Vol.16, pp.235–252

Rowe, G.E. & Margaritis, A.M. (1987). Bioprocess Developments in the Production of Bioinsecticides by *Bacillus thuringiensis. CRC Critical Reviews in Biotechnology.* Vol.6, No.4, pp. 87-127.

Rushed, S.S. & Mulla, M.S. (1989). Factors influencing ingestion of particulate materials by mosquito larvae (Diptera: Culicidae). *Journal of Medical Entomology,* Vol.26, pp. 210-216

Salama, H.S.; Foda, M.S. & Dulmage H.T. (1983). Novel Media for Production of δ-endotoxins form *Bacillus thuringiensis*. *Journal of Invertebrate Pathology,* Vol.41, pp.8-19.

Sen, K.; Honda, G.; Koyama, N.; Nishida, M.; Neki, A.; Sakai, H.; Himeno, M. & Komano, T. (1988). Cloning and nucleotide sequences of the two 130 kDa insecticidal protein genes of *Bacillus thuringiensis* var. *israelensis*. *Agriculture Biology Chemistry*, Vol.52, pp.873-878

Servant, P.; Rosso, M.L.; Hamon, S.; Poncet, S.; Delécluse, A. & Rapport, G. (1999). Production of cry11A and cry11B toxins in *Bacillus sphaericus* confers toxicity towards *Aedes aegypti* and resistant *Culex* populations. *Applied and Environmental Microbiology*, Vol.65, pp.3021–3026.

Sinegre, G.; Gaven, B.; Jullien, J. L. & Crespo, O. (1980). Effectiveness of the serotype H-14 of *Bacillus thuringiensis* against the principal species of man-biting mosquitoes on the Mediterranean coast of France. *Parassitologia* Vol.22, pp.223-231.

Skovmand, O. & Eriksen, A. G. (1993). Field trials of a fizzy tablet with *Bacillus thuringiensis* subsp. *israelensis* in forest spring ponds in Denmark. *Bulletin of the Society for Vector Ecology, Vol.* 18, pp.160-163.

Skovmand, O.; Hoeg, D.; Pedersen, H.S., & Rasmussen T. (1997). Parameters Influencing Potency of *Bacillus thuringiensis* var. *israelensis* Products. *Journal of Economic Entomology* Vol 90, No.2. pp. 361-369.

Smith, A.R. (1982). Effect of strain and medium variation on mosquit production by *Bacillus thuringiensis* var. *israelensis*. *Canadian Journal of Microbiology,* Vol.28, pp.1090-1092.

Tabashnik B.E. (1992). Evaluation of Synergism among *Bacillus thuringiensis* Toxins. *Applied and Environmental Microbiology,* Vol.58, pp.3343-3346

Tanapongpipat, S.; Luxananil, P.; Boonhiang, P.; Chewawiwat, N.; Audtho, M. & Panyim, S. (2003). A plasmid encoding a combination of mosquito-larvicidal genes from *Bacillus thuringiensis* subsp. *israelensis* and *Bacillus sphaericus* confers toxicity against broad range of mosquito larvae when expressed in Gram-negative bacteria. FEMS Microbiology Letters, Vol.228, pp.259–263

Thanabalu, T.; Hindley, J.; Brenner, S.; Oei, C. & Berry, C. (1992). Expression of the mosquitocidal toxins of *Bacillus sphaericus* and *Bacillus thuringiensis* subsp. *israelensis* by recombinant *Caulobacter crescentus*, a vehicle for biological control of aquatic insect larvae. *Applied and Environmental Microbiology,* Vol.58, pp. 905– 910

Thomas, W.E. & Ellar, D.J. (1983) *Bacillus thuringiensis* var. *israelensis* crystal delta-endotoxin : effects on insect and mammalian cells *in vitro* and *in vivo. Journal of Cell Science* Vol.60, pp.181-197.

Thorne, C.B. (1968). Transducing Bacteriophage for *Bacillus cereus*. *Journal Of Virology,* Vol.2, pp. 657-662.

Tungpradubkul, S.; C. Settasatien, & Panyim, S. (1988). The complete nucleotide sequence of a 130 kDa mosquito-larvicidal delta-endotoxin gene of *Bacillus thuringiensis* var. *israelensis*. *Nucleic Acids Research* Vol.16, pp.1637-1638.

Tyanyun, S. & Mulla, M.S. (1999). Field evaluation of new water dispersible granular formulations of *Bacillus thuringiensis* subp. *israelensis* and *Bacillus sphaericus* against Culex mosquito in microcosms. *Journal of the American Mosquito Control Association* Vol 15 pp. 356-361.

Waalwijk, C.; A.; Dullemans, M.; VanWorkum, M.E.S. & Visser, B. (1985). Molecular cloning and the nucleotide sequence of the Mr28,000 crystal protein gene of *Bacillus thuringiensis* subsp. *israelensis*. *Nucleic Acids Research*, Vol.13, pp. 8207-8217

Walsh, J. (1986) River blindness, a gamble pays off. *Science*, Vol.232, pp.922–925.

Ward, E.S.; Ridley, A.R.; Ellar, D.J. & Todd, J.A. (1986). *Bacillus thuringiensis* var. *israelensis* endotoxin. Cloning and expression of the toxin in sporogenic and asporogenic strains of *Bacillus subtilis*. *Journal of Molecular Biology*, Vol.191, pp. 13–22

Ward, E.S., & Ellar, D.J. (1986). *Bacillus thuringiensis* var. *israelensis* delta-endotoxin: nucleotide sequence and characterization of the transcripts in *Bacillus thuringiensis* and *Escherichia coli*. *Journal of Molecular Biology*, Vol.191, pp.1-11

Ward, E. S., & Ellar, D.J. (1987). Nucleotide sequence of a *Bacillus thuringiensis* var. *israelensis* gene encoding a 130 kDa delta-endotoxin. *Nucleic Acids Research*, Vol 15, pp. 7195

Webb, G. (1992) The Onchocerciasis Control Programme. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* Vol. 86, pp.113-114

Wirth, M. C.; Georghiou, G. P.; Federici, B. A. (1997). CytA enables CryIV endotoxins of *Bacillus thuringiensis* to overcome high levels of CryIV resistance in the mosquito, Culex quinquefasciatus. *Proceedings of the National Academy of Sciences,* Vol.94, pp.10536-10540

Wojciech J. J. & Korsten L. (2002). Biological Control of Postharvest Diseases of Fruits. *Annual Review of Phytopathology* Vol. 40, pp. 411-441.

World Health Organization (1999). *World Health Report*. Geneva: World Health Organization.

Wu, D. & Chang, F.N (1985). Synergism in mosquitocidal activity of 26 and 65 kDa proteins from *Bacillus thuringiensis* subsp. *israelensis* crystal. FEBS Letters, Vol.190, pp.232-236

Xu,Y.; Nagai, M.; Bagdasarian, M.; Smith,T.W. & Walker, E.D. (2001). Expression of the *P20* Gene from *Bacillus thuringiensis* H-14 Increases Cry11a Toxin Production and Enhances Mosquito- Larvicidal Activity in Recombinant Gram-Negative Bacteria. *Applied And Environmental Microbiology*, Vol.67, p.p.3010–3015

Wada Y. (1989). Control of Japanese encephalitis vectors. *Southeas*t *Asian journal of tropical medicine and public health,* Vol.20, pp. 623–626

Yamagiwa, M., Sakagawa, K., & Sakai, H. (2004). Functional Analysis of two processed fragments of *Bacillus thuringiensis* cry11A toxin. *Bioscience Biotechnology and Biochemistry*, Vol.68, pp.523–528

Yamamoto, T.; IWatkinson, I.A.; Kim, L.; Sage, M.V.; Stratton, R.; Akande, N.; Li, D.; D.P. & Roe, B.A. (1988). Nucleotide sequence of the gene coding for a 130-kDa mosquitocidal protein of *Bacillus thuringiensis israelensis*. *Gene*, Vol 66, pp.107-120.

Yamamoto, T.; Iizuku, T. & Aronson, J.N. (1983). Mosquitocidal protein of *Bacillus thuringiensis* subsp. *israelensis*: identification and partial isolation of the protein. *Current Microbiology* Vol.9, pp.279-284.

Yu-Tien, L.; Men-Ju, S.; Dar-Der, J.; Wang, W.I.; Chin-Chi, C. & Cheng-Chen, C. (1993). Protection from ultraviolet irradiation by melanin mosquitocidal activity of *Bacillus thuringiensis* var. *israelensis*. *Journal of Invertebrate Pathology,* Vol 62:131-136.

Zeigler, D.R. (1999). *Bacillus thuringiensis Bacillus cereus*. *Bacillus Genetic Stock Center*. Catalog of strains 7th. ed. Vol.2

12

Metabolism of Pyrethroids by Mosquito Cytochrome P450 Enzymes: Impact on Vector Control

Pornpimol Rongnoparut[1], Sirikun Pethuan[1],
Songklod Sarapusit[2] and Panida Lertkiatmongkol[1]
[1]*Department of Biochemistry, Faculty of Science, Mahidol University,*
[2]*Department of Biochemistry, Faculty of Science, Burapha University,*
Thailand

1. Introduction

Cytochrome P450 enzymes (P450s) are heme-containing monooxygenases that catalyze metabolisms of various endogenous and exogenous compounds. These P450s constitute a superfamily of enzymes present in various organisms including mammals, plants, bacteria, and insects. P450 enzymes are diverse and metabolize a wide variety of substrates, but their structures are largely conserved. A universal nomenclature has been assigned to P450 superfamily based on their amino acid sequence homology (Nelson et al., 1996). In eukaryotes, P450 is membrane-bound and in general functions to insert one molecule of oxygen into its substrate, with its heme prosthetic group playing a role in substrate oxidation. This catalytic reaction requires a pair of electrons shuttled from NADPH via the NADPH-cytochrome P450 reductase (CYPOR) enzyme, a P450 redox partner, to target P450s (Ortiz de Montellano, 2005). In contrast in bacteria and mitochondria, ferredoxin reductase and iron-sulfur ferredoxin proteins act as a bridge to transfer reducing equivalent from NAD(P)H to target P450s. In insects, P450s are membrane-bound enzymes that play key roles in endogenous metabolisms (i.e. metabolisms of steroid molting and juvenile hormones, and pheromones) and xenobiotic metabolisms, as well as detoxification of insecticides (Feyereisen, 1999). It becomes evident that P450s are implicated in pyrethroid resistance in insects.

Insecticides form a mainstay for vector control programs of vector-borne diseases. However intensive uses of insecticides have led to development of insecticide resistance in many insects thus compromising success of insect vector control. In particular pyrethroid resistance has been found widespread in many insects such as house flies, cockroaches, and mosquitoes (Acevedo et al., 2009; Awolola et al., 2002; Cochran, 1989; Hargreaves et al., 2000; Jirakanjanakit et al., 2007). Two major mechanisms have been recorded responsible for insecticide resistance, which are alteration of target sites and metabolic resistance (Hemingway et al., 2004). Metabolic resistance is conferred by increased activities of detoxification enzymes such as P450s, non-specific esterases (Hemingway et al., 2004; Price, 1991). Initial approaches to detect involvement of detoxification mechanisms in metabolic resistance are to compare activities of detoxification enzymes between resistant and

susceptible insect strains, and by identification of corresponding genes that display higher expression level in resistant insects (Bautista et al., 2007; Chareonviriyaphap et al., 2003; Tomita et al., 1995; Yaicharoen et al., 2005). Examinations in various insects such as house fly, cotton ballworm, and mosquito have implicated involvement of up-regulation of different P450 genes in pyrethroid resistance (Liu & Scott, 1998; Müller et al., 2007; Ranasinghe & Hobbs, 1998; Rodpradit et al., 2005; Tomita et al., 1995). Such P450 overexpression has been assumed constituting a defense mechanism against insecticides and responsible for insecticide resistance, presumably by virtue of enhanced insecticide detoxification.

Recent advanced methods employing microarray-based approach, when genomic sequence information for insects is available, have identified multiple genes involved in pyrethroid resistance in mosquitoes. Genes in CYP6 family, in particular, are reported to have an implication in insecticide resistance. In *Anopheles gambiae* malaria vector, microarray analyses reveal that several CYP6 P450 genes could contribute to pyrethroid resistance, these include CYP6M2, CYP6Z2 and CYP6P3 (Djouaka et al., 2008; Müller et al., 2007). These genes were observed up-regulated in pyrethroid resistant mosquitoes (Müller et al., 2008; Stevenson et al., 2011). CYP6M2 and CYP6P3 have shown ability to bind and metabolize pyrethroids, on the other hand CYP6Z2 is able to bind pyrethroids but does not degrade pyrethroids (Mclauglin et al., 2008). Genetic mapping of genes conferring pyrethroid resistance in *An. gambiae* also supports involvement of CYP6P3 in pyrethroid resistance (Wondji et al., 2007). Up-regulation of CYP6 genes has also been found in other resistant insects, for instance CYP6BQ9 in pyrethroid resistant *Tribolium castaneum* (Zhu et al., 2010), CYP6D1 in *Musca domestica* that is able to metabolize pyrethroids (Zhang & Scott, 1996), and CYP6BG1 in pyrethroid resistant *Plutella xylostella* (Bautista et al., 2007). In *T. castaneum* knockdown of CYP6BQ9 by dsRNA resulted in decreased resistance to deltamethrin (Zhu et al., 2010). Similar finding has been observed for CYP6BG1 in permethrin resistant *P. xylostella*, supporting the role of overexpression of these CYP6 genes in pyrethroid resistance (Bautista et al., 2009). In *An. minimus* mosquito, CYP6AA3 and CYP6P7 are upregulated and possess activities toward pyrethroid degradation (Duangkaew et al., 2011b; Rongnoparut et al., 2003).

2. Cytochrome P450 monooxygenase (P450) and NADPH-cytochrome P450 reductase (CYPOR) enzymes isolated from *An. minimus*

In this chapter, we focus on investigation of the P450s that have been shown overexpressed in a laboratory-selected pyrethroid resistant *An. minimus* mosquito. We describe heterologous expression of the overexpressed P450s in baculovirus-mediated insect cell expression system and characterization of their catalytic roles toward pyrethroid insecticides. Tools utilized in functional investigation of *An. minimus* P450s have been developed and described. In parallel the *An. minimus* CYPOR has been cloned and protein expressed via bacterial expression system. Amino acid sequence of *An. minimus* CYPOR is intrigue in that several important residues that might play role in its functioning as P450 redox partner are different from those of previously reported enzymes from mammals and house fly. The *An. minimus* CYPOR is different in enzymatic properties and kinetic mechanisms from other CYPORs. In this context we speculate that *An. minimus* CYPOR could influence electron delivery to target mosquito P450 enzymes, and could act as a rate-limiting step in P450-mediated metabolisms. These results together could thus gain an

insight into pyrethroid metabolisms in this mosquito species and knowledge obtained could contribute to strategies in control of mosquito vectors.

An. minimus is one of malaria vectors in Southeast Asia, including Thailand, Loas, Cambodia and Vietnam. We previously established a deltamethrin-selected mosquito strain of *An. minimus* species A, by exposure of subsequent mosquito generations to LD_{50} and LT_{50} values of deltamethrin (Chareonviriyaphap et al., 2002). Biochemical assays suggested that deltamethrin-resistant *An. minimus* predominantly employ P450s to detoxify pyrethroids (Chareonviriyaphap et al., 2003). We next set out on isolation of P450 genes that have a causal linkage in conferring deltamethrin resistance in this mosquito species. Using reverse-transcribed-polymerase chain reaction (RT-PCR) in combination with degenerate PCR primers whose sequences were based on CYP6 conserved amino acids, we have isolated CYP6AA3, CYP6P7, and CYP6P8 complete cDNAs from deltamethrin-resistant *An. minimus* (Rongnoparut et al., 2003). The three genes showed elevated transcription level in deltamethrin resistant populations compared to the parent susceptible strain. We found that fold of mRNA increase of CYP6AA3 and CYP6P7 is correlated with increase of resistance during deltamethrin selection. However, this correlation was not observed for CYP6P8 (Rodpradit et al., 2005). The three mosquito P450s could thus be used as model enzymes for characterization of their metabolic activities toward insecticides and possibly for future development of tools for mosquito vector control. This can be accomplished by determining whether they possess catalytic activities toward pyrethroid insecticides, thus assuming a causal linkage of overexpression and increased pyrethroid detoxification leading to pyrethroid resistance. Equally important, elucidating properties of the *An. minimus* CYPOR and its influential role in P450 system is beneficial for understanding of P450 metabolisms of this mosquito species.

2.1 *In vitro* insecticide metabolisms

We have heterologously expressed CYP6AA3, CYP6P7, and CYP6P8 in *Spodoptera frugiperda* (*Sf*9) insect cells via baculovirus-mediated expression system. The expression procedure employed full-length CYP6AA3, CYP6P7, and CYP6P8 cDNAs as templates to produce recombinant baculoviruses, and subsequently infected *Sf*9 cells for production of P450 proteins. RT-PCR amplification and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis were performed to verify expression of P450 mRNAs and proteins in the infected *Sf*9 cells. Expression of CYP6AA3, CYP6P7, and CYP6P8, each is predominantly detected in membrane fractions of infected cells after 72 hours of infection, with expected molecular size of approximately 59 kDa detected on SDS-PAGE (Kaewpa et al., 2007; Duangkaew et al., 2011b). The expressed proteins display CO-reduced difference spectrum of a characteristic peak at 450 nm (Omura & Sato, 1964). Total P450 content obtained from baculovirus-mediated expression of CYP6AA3, CYP6P7, and CYP6P8 ranges from 200 to 360 pmol/mg membrane protein. The expressed CYP6AA3, CYP6P7, and CYP6P8 proteins were used in enzymatic reaction assays testing against pyrethroids and other insecticide groups. Knowledge of the metabolic profile of these P450s could give us insight into functioning of these P450s within mosquitoes towards insecticide metabolisms, i.e. how mosquitoes detoxify against a spectrum of insecticide classes through P450-mediated metabolisms.

In enzymatic assay, each P450 in the reaction was performed in the presence of NADPH-regenerating system and was reconstituted with *An.minimus* CYPOR (Kaewpa et al., 2007), as CYPOR is required to supply electrons to P450 in the reaction cycle. Insecticide

metabolism was determined by detection of disappearance of insecticide substrate at different times compared with that present at time zero as previously described (Booseupsakul et al., 2008). This time course degradation was detected through HPLC analysis. Table 1 summarizes enzyme activities of CYP6AA3 and CYP6P7 toward insecticides and metabolites detected. Insecticides that were tested by enzyme assays were type I pyrethroids (permethrin and bioallethrin), type II pyrethroids (deltamethrin, cypermethrin, and λ-cyhalothrin), organophosphate (chlorpyrifos), and carbamate (propoxur). Additional insecticides (bifenthrin, dichlorvos, fenitrothion, temephos, and thiodicarb) belonging to these four insecticide classes were tested by cytotoxicity assays (see Section 2.3). Chemical structures of these insecticides are shown in Fig. 1.

Insecticides	CYP6AA3 Activity (metabolites)	CYP6P7 Activity
Type I pyrethroids		
Bioallethrin	-	-
Permethrin	+ (1 major unknown product)	+, ND
Type II pyrethroids		
Cypermethrin	+ (3-phenoxybenzaldehyde and 2 unknown products)	+, ND
Deltamethrin	+ (3-phenoxybenzaldehyde and 2 unknown products)	+, ND
λ - Cyhalothrin	+, ND	-
Organophosphate		
Chlorpyrifos	-	-
Carbamate	-	-
Propoxur	-	-

Table 1. Presence (+) and absence (-) of P450 activities in insecticide degradation and metabolites obtained. ND, products not determined

The results shown in Table 1 demonstrate that CYP6AA3 and CYP6P7 share overlapping metabolic profile against both type I and II pyrethroids, while no detectable activity was observed toward chlorpyrifos and propoxur (Duangkaew et al., 2011b), nor in the presence of piperonyl butoxide (a P450 inhibitor). Differences in activities of both enzymes could be noted, for CPY6AA3 could metabolize λ-cyhalothrin while CYP6P7 did not display activity against λ-cyhalothrin. For CYP6P8 we initially detected absence of pyrethroid degradation activity, further tests using cytotoxicity assays described in Section 2.3 suggest that CYP6P8 is not capable of degradation of pyrethroids, organophosphates and carbamates.

Determination of products obtained from CYP6AA3-mediated pyrethroid degradations using GC-MS analysis reveal multiple products for type II pyrethroid cypermethrin degradation and for earlier described deltamethrin metabolism (Boonseupsakul et al., 2008). These products were 3-phenoxybenzyaldehyde and two unknown products with chloride and bromide isotope distribution derived from cypermethrin and deltamethrin metabolisms, respectively. In contrast there was only one unknown product that was predominantly detected from CYP6AA3-mediated permethrin (type I pyrethroid) degradation, with mass spectrum profile showing characteristic chloride isotope distribution of permethrin-derived compound. Unlike cypermethrin and deltamethrin metabolisms, we did not obtain 3-phenoxybenzaldehyde from permethrin degradation (Boonseupsakul, 2008).

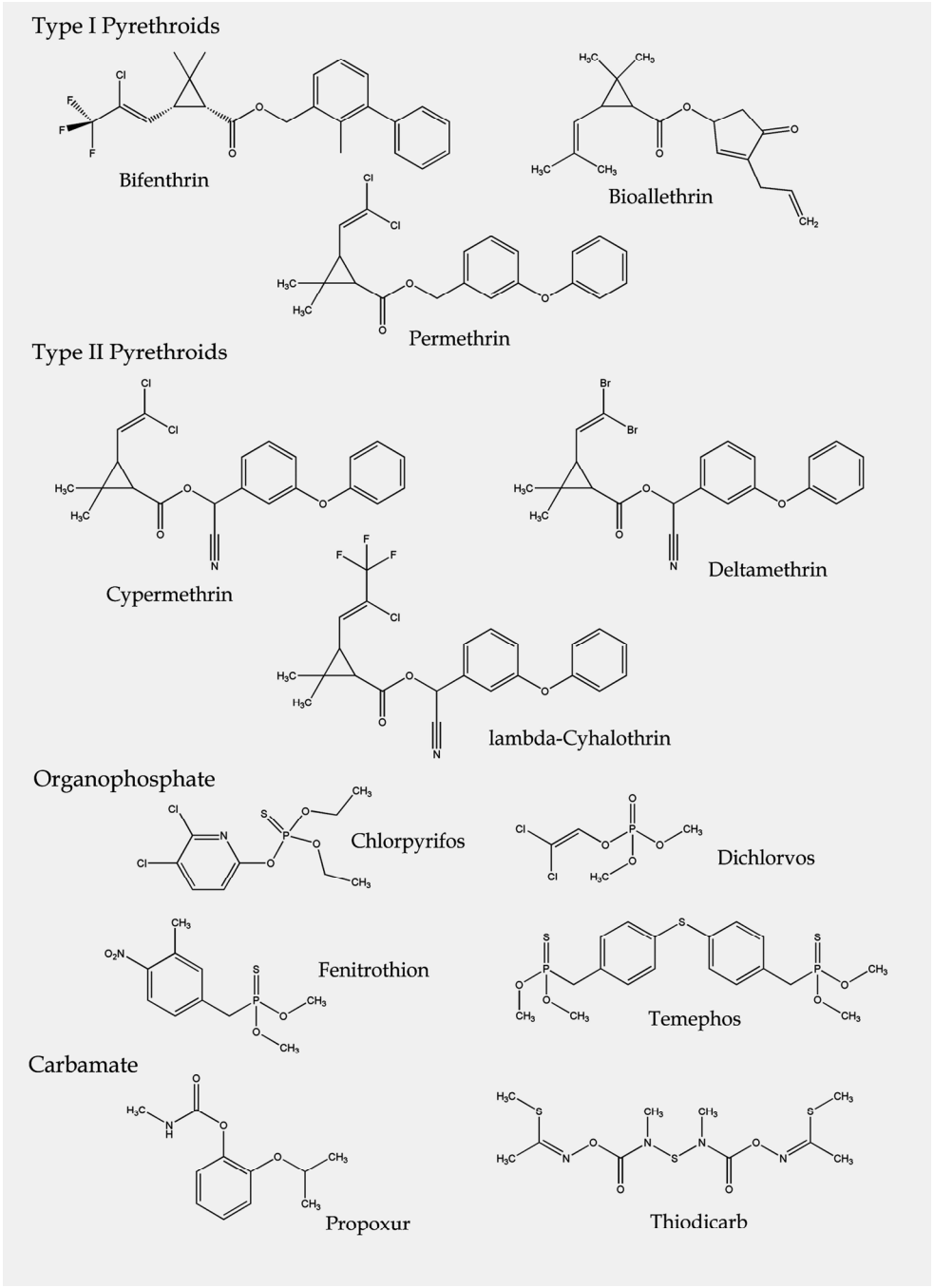

Fig. 1. Chemical structures of insecticides used in the study.

Type I and type II pyrethroids are different by the presence of cyano group (see Fig. 1). Thus our results implicate that presence of cyano group may play role in CYP6AA3-mediated pyrethroid degradations resulting in detection of 3-phenoxybenzaldehyde, possibly through oxidative cleavage reaction. In *An. gambiae* CYP6M2-mediated deltamethrin metabolism and house fly CYP6D1-mediated cypermethrin metabolism, 4′-hydroxylation of deltamethrin and cypermethrin is the major route of their metabolisms since 4′-hydroxylation products were predominantly detected (Stevenson et al., 2011; Zhang & Scott, 1996). The 4′-hydroxylation and 3-phenoxybenzaldehyde products have been observed in *in vitro* pyrethroid metabolisms mediated by mammalian microsomal enzymes (Shono et al., 1979). The absence of detection of 3-phenoxybenzaldehyde in CYP6AA3-mediated permethrin degradation could be predicted that the reaction underwent monooxygenation of permethrin.

2.2 Characterization of CYP6AA3 and CYP6P7 enzymes

As described, both CYP6AA3 and CYP6P7 enzymes have enzymatic activities against pyrethroid insecticides and their metabolic profiles are different. Kinetics and inhibition studies further support their abilities to metabolize pyrethroids, however with different enzyme and kinetic properties that influence substrate and inhibitor selectivity. Such knowledge could have an implication in pyrethroid detoxification in *An. minimus* mosquito, for example how the two P450s redundantly metabolize overlapping sets of pyrethroids. Alongside investigation of pyrethroid metabolisms, we examined their activities toward fluorescent compounds for development of rapid enzymatic assays. Finally we performed cell-based MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) cytotoxicity assays for further determination of substrates and inhibitors of both P450 enzymes as reported herein.

2.2.1 Determination of enzyme kinetics of CYP6AA3 and CYP6P7 enzymes

We recently reported kenetic paremeters for CYP6AA3 and CYP6P7 enzymes (Duangkaew et al., 2011b). Kinetic results reveal that CYP6AA3 has preference in binding to and has higher rate in degradation of permethrin type I pyrethroid than type II pyrethroids (K_m values toward permethrin, cypermethrin, deltamethrin, and λ-cyhalothrin of 41.0 ± 8.5, 70.0 ± 7.1, 80.2 ± 2.0, and 78.3 ± 7.0 μM, respectively and V_{max} values of 124.2 ± 1.2, 40.0 ± 7.1, 60.2 ± 3.6, and 60.7 ± 1.1 pmol/min/pmol P450, respectively). In contradictory CYP6P7 does not have preference for type of pyrethroids (K_m values toward permethrin, cypermethrin, and deltamethrin of 69.7 ± 10.5 , 97.3 ± 6.4, and 73.3 ± 2.9, respectively and V_{max} values of 65.7 ± 1.6, 83.3 ± 7.6, and 55.3 ± 5.7 pmol/min/pmol P450, respectively) and does not metabolize λ-cyhalothrin. Thus although both enzymes are comparable in terms of capability to metabolize pyrethroids *in vitro*, their kinetic values are different. Enzyme structure could account for their differences in kinetic properties and substrate preference.

Since there has been no known crystal structure available for insect P450s, we initially constructed homology models for CYP6AA3, CYP6P7, and CYP6P8 in an attempt to increase our understanding of molecular mechanisms underlying their binding sites toward insecticide substrates and inhibitors. The three enzyme models are different in geometry of their active-site cavities and substrate access channels. Upon docking with various insecticide groups, results of its active site could predict and explain metabolic behavior toward pyrethroid, organophosphate, and carbamate insecticides (Lertkiatmongkol et al., 2011). These results suggest that differences in metabolic activities among P450 enzymes in

insects could be attributed to structural differences resulting in selectivity and different enzymatic activities against insecticides.

In human, CYP2C8, CYP2C9, CYP2C19, and CYP3A4 have been reported abilities to metabolize both type I and II pyrethroids (Godin et al., 2007; Scollon et al., 2009). The preference for type I pyrethroid in CYP6AA3 is similar to human CYP2C9 and CYP2C19, while similar metabolic activity toward both types of pyrethroids found for CYP6P7 is similar to that of human CYP2C8 enzyme (Scollon et al., 2009). Nevertheless efficiency of CYP6AA3 and CYP6P7 in deltamethrin degradation is 5- to 10-fold less effective than human CYP2C8 and CYP2C19. It is noteworthy that more than one P450s residing within an organism can metabolize pyrethroids as described for human and mosquito, multiple rat P450s are also found capable of pyrethroid metabolisms (Scollon et al., 2009). When comparing to *An. gambiae* CYP6P3, both CYP6AA3 and CYP6P7 possess at least 10 fold higher K_m than CYP6P3, but V_{max} values of both *An. minimus* CYP6AA3 and CYP6P7 are at least 20 fold higher (Müller et al., 2008). Higher values of K_m and V_{max} of CYP6AA3 and CYP6P7 than those values of *An. gambiae* CYP6M2 (Stevenson et al., 2011) are also observed.

2.2.2 CYP6AA3 and CYP6P7 are inhibited differently by different compounds

To obtain a potential fluorogenic substrate probe for fluorescent-based assays of CYP6AA3 and CYP6P7, we previously screened four resorufin fluorogenic substrates containing different alkyl groups (Duangkaew et al., 2011b) and results in Table 2 suggest that among test compounds, benzyloxyresorufin could be used as a fluorescent substrate probe since both CYP6P7 and CYP6AA3 could bind and metabolize benzyloxyresorufin with lowest K_m (values of 1.92 for CYP6AA3 and 0.49 for CYP6P7) and with highest specific activities (Duangkaew et al., 2011b). The assays of benzyloxyresorufin-*O*-debenzylation activity were further used for inhibition studies of both mosquito enzymes.

Specific activity (pmole resorufin/min/pmole P450)		
Compounds	CYP6AA3	CYP6P7
Benzyloxyresorufin	6.81 ± 0.65	4.99 ± 0.74
Ethyloxyresorufin	2.88 ± 0.21	3.61 ± 0.17
Methyloxyresorufin	0.02 ± 0.01	-
Penthyloxyresorufin	0.01 ± 0.01	-

Table 2. Specific activities of CYP6AA3 and CYP6P7 toward resorufin derivatives.

Using fluorescence-based assays, we could initially determine what compound types that give mechanism-based inhibition pattern by pre-incubation of enzyme with various concentrations of test inhibitors in the presence or absence of NADPH for 30 min before addition of substrates and IC_{50} values have been determined as described (Duangkaew et al., 2011b). As known, mechanism-based inactivation inhibits enzyme irreversibly, rendering this mechanism of inhibition more efficient than reversible inhibition. Nevertheless information on mode of inhibition for inhibitors is potential for understanding of catalytic nature of enzymes. We thus determined mode of inhibition for all compounds tested. As shown in Table 2, the compounds we have tested are phenolic compounds and their chemical structures are shown in Fig. 2.

It is apparent that none of test flavonoids and furanocoumarins shows mechanism-based inhibition pattern, but piperonyl butoxide (PBO) and piperine that are methylenedioxyphenyl compounds show NADPH-dependent mechanism-based inhibition activities against both

enzymes. Piperine has been commonly found in *Piper sp.* plant extracts, it possesses acute toxicity to mammals (Daware et al., 2000). Inhibition results shown in Table 3 also elucidate that α-naphthoflavone displayed strongest inhibitory effect. Its inhibition pattern suggests that α-naphthoflavone uncompetitively inhibit both enzymes by binding to CYP6AA3– and CYP6P7-benzyloxyresorufin complex. Moreover, a difference was noted for xanthotoxin as it uncompetitively inhibits CYP6AA3 but mixed-type inhibited CYP6P7. Thus inhibition results together with different metabolic profiles thus confirm that CYP6AA3 and CYP6P7 have different enzyme properties. We thus also tested crude extracts of two plants (*Citrus reticulate* and *Stemona spp.*) that were reported containing phenolic compounds (Kaltenegger et al., 2003; Jayaprakasha et al., 1997) and are found in Thailand. Initial results suggest that compounds within both plants may not possess mechanism-based activities against CYP6AA3 and CYP6P7, and both extracts did not inhibit both enzymes as efficient as flavonoids and methylenedioxyphenyl compounds.

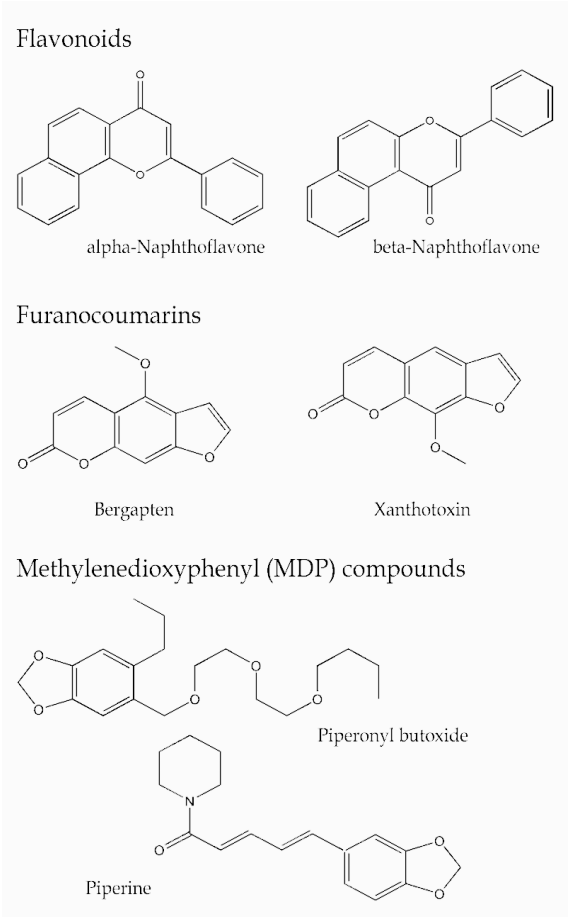

Fig. 2. Chemical structures of different compound types used for inhibition assays of mosquito P450s.

Inhibitor	Inhibition type (K_i, μM)		IC_{50} (M)			
	CYP6AA3	CYP6P7	CYP6AA3 pre-incubation		CYP6P7 pre-incubation	
			w/o NADPH	w/ NADPH	w/o NADPH	w/ NADPH
Flavonoids						
α-Naphthoflavone	Uncompetitive (0.84)	Uncompetitive (2.02)	0.37 ± 0.06	0.38 ± 0.06	2.90 ± 0.27	3.03 ± 0.45
-Naphthoflavone	ND	ND	19.22 ± 3.13[a]	34.44 ± 5.95[a]	17.25 ± 3.67	33.35 ± 9.90
Furanocoumarins						
Xanthotoxin	Uncompetitive (52.45)	Mixed-type (47.14)	51.04 ± 2.15	52.17 ± 2.86	33.77 ± 3.54[a]	78.93 ± 10.04[a]
Bergapten	Mixed-type (93.27)	Mixed-type (65.59)	93.77±10.87[a]	170.3 ± 16.88[a]	52.76 ± 6.77[a]	114.0 ± 11.81[a]
Methylenedioxyphenyl (MDP) compounds						
Piperine	Mechanism-based (ND)	Mechanism-based (ND)	15.26± 1.21[a]	4.86 ± 0.79[a]	52.86 ± 6.92[a]	3.48 ± 0.36[a]
Piperonyl butoxide (PBO)	Mechanism-based (ND)	Mechanism-based (ND)	9.91 ± 0.81[a]	4.04 ± 0.31[a]	31.77 ± 3.21[a]	16.22 ± 1.81[a]
Crude Extracts						
Citrus reticulata	ND	ND	236.1± 32.6	234.9 ± 9.54	116.4 ± 16.54	141.1 ± 15.1
Stemona spp.	ND	ND	56.11 ± 7.05	63.91 ± 5.2	71.77 ± 5.73[a]	105.7 ±10.18[a]

Table 3. Mode of inhibition and inhibition constants of CYP6P7- or CYP6AA3-benzyloxyresorufin-*O*-debenzylation activities of flavonoids, furanocoumarins, and MDP compounds (Duangkaew et al., 2011b). Crude plant extracts reported herein are ethanolic extracts. Values marked with 'a' are significantly different between reactions with (w/) and without (w/o) NADPH. ND, not determined.

2.3 Use of cell-based MTT cytotoxicity assays to determine insecticide substrates and inhibitiors of *An. minimus* P450 enzymes

Since *in vitro* reconstitution system demonstrated CYP6AA3 and CYP6P7 enzymatic activities against pyrethroids, further investigation of the ability of CYP6AA3 and CYP6P7 enzymes to eliminate pyrethroid toxicity from cells was assessed in P450-infected *Sf*9 cells. This can be accomplished because other than targeting on sodium channels of nervous system, pyrethroids possess toxic effects on cells such as inhibition of cell mitochondrial complex I or causing DNA damage and cell death (Gassner et al., 1997; Patel et al., 2007; Naravaneni & Jamil, 2005). Similar cell death and cytotoxic to cells caused by organophosphates and carbamate insecticides have also been reported (Maran et al., 2010; Schmuck & Mihail, 2004). This is supported by that we previously observed cytotoxic effects of deltamthrin on insect *Sf*9 cells. When using *Sf*9 cells that express CYP6AA3 in MTT assays, cell mortality was drastically decreased in the presence of insecticides due to degradation of deltamethrin by CYP6AA3 and thus posing cytoprotective role on *Sf*9 cells (Boonseupsakul et al., 2008). Use of insect cells to test for toxicity effects of compounds such as fungal metabolites (Fornelli et al., 2004) and pyridalyl insecticide (Saito et al., 2005) has been previously reported. Moreover, insect cells expressing P450 have also been successfully used to test detoxification capability of enzyme against cytotoxic xenochemicals (Grant et al., 1996; Greene et al., 2000). In this context, we used MTT assays to determine insecticide detoxification by P450 expressed in *Sf*9 cells. Insecticides tested were pyrethroids (deltamethrin, permethrin, cypermethrin, bifenthrin, bioallethrin and λ-cyhalothrin), organophosphates (chlorpyrifos, dichlorvos, fenitrothion and temephos), carbamates (thiodicarb and propoxur). Various concentrations (1-500 μM) of insecticides were used for determination of cytotoxic effect of insecticides toward CYP6AA3-, CYP6P7-, and CYP6P8-expressing cells and compared to the control parent *Sf*9 cells. Cell viability of insecticide treated cells was measured by MTT assay as previously described (Boonseupsakul et al., 2008) and plotted against insecticide concentrations. The LC_{50} value of each insecticide was subsequently evaluated and obtained from each plot. Table 4 summarizes LC_{50} values of insecticides against *Sf*9 cells and cells with expression of P450s.

We observed that pyrethroids, organophosphates and carbamates are toxic to *Sf*9 parent cells. Since LC_{50} values of permethrin, bifenthrin, cypermethrin, and deltamethrin against CYP6AA3- and CYP6P7-expressing cells were approximately 4- to 19-folds significantly greater than those from parent *Sf*9 cells, these values imply that CYP6AA3 and CYP6P7 enzymes could cytoprotect *Sf*9 cells from pyrethroid toxicity. Conversely there was no significant difference of IC_{50} values between cells treated with organophosphate (chlorpyrifos, fenitrothion and temephos), carbamates (thiodicarb and propoxur) and bioallethrin pyrethroid insecticide, suggesting that expression of P450s did not cytoprotect cells from these insecticides. In addition CYP6P8 did not cytoprotect *Sf*9 cells against insecticides tested. It should be noted that LC_{50} value of λ-cyhalothrin in CYP6AA3-expressing cells was significantly greater than *Sf*9 parent cells, but not in CYP6P7-expressing cells. These results from MTT cytotoxicity assays are thus in agreement with *in vitro* enzymatic assays as described in Section 2.1. Thus abilities to cytoprotect against insecticide toxicity in infected *Sf*9 cells are due to P450-mediated enzymatic activity toward insecticides of CYP6AA3 and CYP6P7 (Duangkaew et al., 2011a). Together with *in vitro* enzymatic and cytotoxicity assays, we can conclude that both CYP6AA3 and CYP6P7 share metabolic activities against pyrethroids, but both enzymes play no role in degradations of organophosphates and carbamates. The results suggest that CYP6P8 plays no role in

degradation of insecticides tested in this report. Moreover, such cytotoxicity results implicate that the method could also be applied for primary screening of compounds that have an inhibitory effect towards CYP6AA3 and CYP6P7, as well as P450 enzymes that possess enzymatic activities against these insecticides.

Insecticides	LC_{50} (μM)			
	*Sf*9	CYP6AA3	CYP6P7	CYP6P8
Pyrethroids				
Bioallethrin[b]	30.6 ± 2.1	32.7 ± 2.4	23.3 ± 3.9	29
Permethrin[b]	42.7 ± 1.8	406.7 ± 21.5[a]	214.7 ± 48.8[a]	78
Bifenthrin	45 ± 7.6	210 ± 12.4[a]	135 ± 51[a]	45
Cypermethrin[b]	21.8 ± 0.5	192.7 ± 30.4[a]	216.7 ± 21.4[a]	25
Deltamethrin[b]	27.5 ± 9.2	285.0 ± 27.8[a]	379.5 ± 21.9[a]	10
λ-Cyhalothrin[b]	38.4 ± 4.3	133.3 ± 37.5[a]	42.0 ± 1.8	ND
Organophosphates				
Chlorpyrifos[b]	40.3 ± 6.5	56.3 ± 8.5	41.7 ± 2.8	60
Fenitrothion	25.0 ± 5.3	30.0 ± 6.4	ND	25
Temephos	11.0 ± 3.9	19.0 ± 7.5	ND	ND
Dichlorvos	32.0 ± 8.9	39.0 ± 6.4	ND	ND
Carbamates				
Propoxur[b]	4.0 ± 6.6	4.7 ± 0.3	3.6 ± 0.2	ND
Thiodicarb	28.6 ± 2.3	29.2 ± 4.7	ND	ND

Table 4. Cytotoxicity effects by insecticides on P450-infected cells and the parent SF9 cells using MTT assays. Values reported for CYP6P8 were average obtained from experiments performed in duplicate. Those marked with 'a' were significantly different from parent *Sf*9 cells and those marked with 'b' were reported in Duangkaew et al, 2011a. ND, not determined.

To test whether inhibitors can be screened, MTT assays were performed with P450-expressing cells treated with 100 μM deltamethin in the presence or absence of each test inhibitor. Concentrations of test inhibitory compounds were those of approximately LC_{20} values pre-determined by MTT assays on *Sf*9 cells. In cell-based inhibition assays, cell viability was determined upon co-incubation of test compound and deltamethrin, and normalized with viability of cells treated with test compound without deltamethrin. Inhibition experiments were performed with control *Sf*9 cells in the same manner as CYP6AA3-expressing cells and percent cell viability was plotted against test inhibitor concentrations, and results demonstrated that cell viability of parent *Sf*9 cells was not affected by test compounds (data not shown).

The results shown in Fig. 3 indicate that cell viability of CYP6AA3-expressing cells was decreased upon increasing concentration of test inhibitors. Piperine, piperonyl butoxide, and α-naphthoflavone could inhibit cytoprotective activity of CYP6AA3 more than xanthotoxin. This is thus in compliance with *in vitro* enzymatic inhibition assays, although piperonyl butoxide showed more potential than α-naphthoflavone in inhibiting cytoprotective activity of CYP6AA3. Cell permeability of test compounds could be accounted for differences of cell-based MTT and *in vitro* enzymatic assays. The results however indicate usefulness of cells expressing P450 enzymes to primarily screen for P450 substrates and inhibitors. Our results indicated that PBO and piperine could inhibit P450s

and possess synergistic actions against deltamethrin cytotoxicity in Sf9 cells expressing P450. PBO has been used as pyrethroid synergist to enhance pyrethroid toxicity, as it can bind to P450s thereby inhibiting P450 activity (Fakoorziba et al., 2009; Kumar et al., 2002; Vijayan et al., 2007). Unfortunately PBO has been reported acutely toxic to mammals (Cox, 2002; Okamiya et al., 1998).

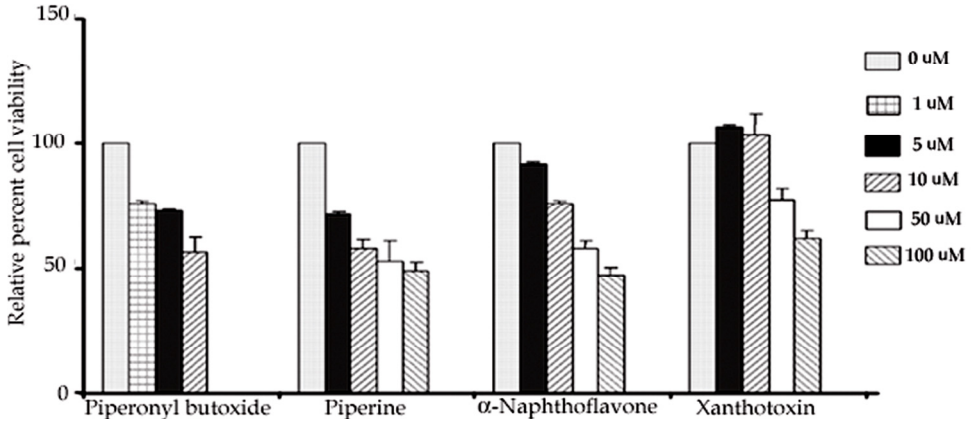

Fig. 3. Inhibition effect of test compounds against cell-based CYP6AA3-mediated deltamethrin detoxification measured by MTT assays.

2.4 *An. minimus* CYPOR and its possible role in regulation of P450-reaction cycle

The NADPH-Cytochrome P450 oxidoreductase (CYPOR) enzyme is a member of di-flavin enzymes that transfers electrons, one by one, from NADPH through FAD and FMN to target enzymes to fulfill functioning of various cytochrome P450 enzymes as well as other enzymes (Murataliev et al., 2004). Other members of this class are those containing a flavoprotein subunit, such as nitric oxide synthase, sulfite reductase, methionine synthase reductase and protein NR 1. Detailed biochemical and structural studies of rat CYPOR reveal several conserved structural domains existed in this enzyme class, these are membrane-bound, FMN-binding, connecting, and FAD/NADPH binding domains (Wang et al., 1997).

The *An. minimus* CYPOR has been cloned and expressed in *E. coli*, and CYPOR could support CYP6AA3- and CYP6P7-mediated pyrethroid metabolisms *in vitro* (Duangkaew et al., 2011b; Kaewpa et al., 2007). However its expression has been of poor yield as a result of inclusion bodies formation. An attempt to obtain soluble protein by deletion of the first 55 amino acid residues comprising of membrane binding region (*Δ55AnCYPOR*) has been successful (Sarapusit et al., 2008). However the protein could not be purified by 2′5′-ADP affinity column, indicating that NADPH binding capacity of mosquito CYPOR is low and this is different from CYPORs of other organisms such as rat and human (Sarapusit, 2009). Low binding affinity to 2′5′-ADP affinity column has also been recently reported in *An. gambiae* CYPOR (Lian et al., 2011). Only under specific condition was *Δ55AnCYPOR* successfully expressed and purified to homogeneity by a combination of Ni^{2+}NTA-affinity chromatography and G200-gel filtration chromatography (Sarapusit et al., 2008). Moreover both purified full-length (*flAnCYPOR*) and membrane-deleted *Δ55AnCYPOR* proteins readily lose FAD and FMN cofactors, they undergo

aggregation and are unstable compared to rat and human CYPORs (Sarapusit et al., 2008, 2010). While supplementation of FAD could increase activity of both full-length and membrane-deleted forms, FMN supplementation could increase activity of full-length form only (Sarapusit et al., 2008, 2010). This behavior is different from membrane-deleted soluble CYPORs of rat and human in which exogenous FMN is readily incorporated into its FMN-binding site (Döhr et al., 2001; Shen et al., 1989). Due to loss of flavin cofactors and instability of *An. minimus* CYPOR, we have identified two key amino acids (Leu86 and Leu219 in FMN binding domain) by amino sequence alignment and shown that mutations of the two leucine residues into conserved phenylalanine residues that are found conserved among other CYPORs could rescue loss of FAD cofactor and increase protein stability of mosquito CYPOR (Sarapusit et al., 2008, 2010). These mutations do not affect kinetic mechanism and constants of enzyme. Double mutations of leucine to the conserved phenylalanine (L86F/L219F) in full-length *flAnCYPOR*, but not in *Δ55AnCYPOR*, could increase binding of FMN and increase CYP6AA3-mediated pyrethroid metabolism (Sarapusit et al., 2010), indicating that membrane-bound region of *An. minimus* CYPOR could influence both structural folding of FMN domain and mediation of P450 catalysis (Murataliev et al., 2004; Wang et al., 1997).

The enzyme activity and kinetic mechanism of *flAnCYPOR* using cytochrome c as substrate are ionic strength dependent, with its mechanism following random Bi-Bi mechanism at low ionic strength and non-classical two-side Ping-Pong at high ionic strength. These mechanisms are different from rat, human, and house fly CYPORs (Murataliev et al., 2004; Sem & Kasper, 1994, 1995). In addition, *flAnCYPOR* could use extra flavins as additional substrates in which FAD binds at FAD/NADPH domain and FMN binds at FMN domain (as depicted in Fig. 4), resulting in an increase in its rate of electron transfer in CYP6AA3-mediated pyrethroid degradation (Sarapusit et al., 2010).

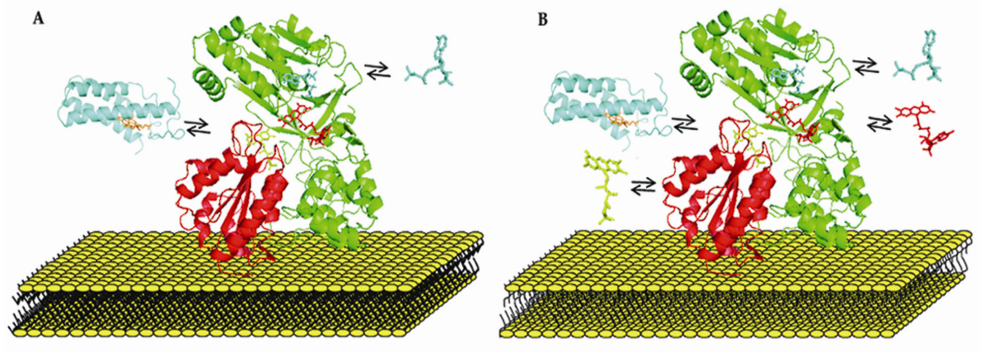

Fig. 4. Schematic representation of enzymatic reactions of CYPOR enzymes. CYPOR enzymes are represented in cartoon model of which FMN domain is in red color and FAD/NADPH domain is in green. Cofactors are represented in the stick mode; FMN is yellow colored, FAD is red, and $NADP^+$ is cyan (rat CYOR: pdb code 1AMO). The cytochrome *c* substrate (cytychrome *c*: pdb code 1BBH) is in cyan cartoon model with an orange heme group residing at the center.

In Figure 4, panel A illustrates common CYPOR (such as rat, human CYPORs) to which NADPH and cytochrome *c* substrate separately binds FAD/NADPH and FMN domains, while in panel B, *flAnCYPOR* could use extra flavins as additional substrates to which FAD

cofactor binds FAD/NADPH domain and FMN cofactor binds FMN domain. We thus speculate that *An. minimus* mosquito uses CYPOR in regulation of P450-mediated metabolisms, since it supplies electrons to a collection of P450s within the cell. Although structural basis for loose binding of flavin cofactors in *An. minimus* CYPOR is not known, it is conceivable that its distinct property that adopt extra flavins as substrates may render the enzyme ability to regulate electron transfer to target mosquito enzymes.

3. Conclusion

The results of this study on CYP6AA3 and CYP6P7 could lay groundwork into an understanding of the mechanisms that control substrates and reaction selectivity of both P450 enzymes, thereby increase an understanding of P450-mediated resistance mechanisms to various pesticides. The kinetic values, metabolic profile of pyrethroid insecticide metabolisms and inhibition patterns by different inhibitors of CYP6AA3 are different from CYP6P7. Future approach could aim at the strategy involving finding a collection of substrates together with structural models and mutation analyses of CYP6AA3 and CYP6P7 that affect specific P450 catalysis. Moreover, characterizing inhibitors and inhibition mechanisms of large collection of compounds with known chemical structures against CYP6AA3 and CYP6P7 enzymes could give insight into an understanding of mechanisms of cytochrome P450s that metabolize pyrethroids. It is conceivable that CYP6P8 does not play role in detoxification of pyrethroid, organophosphate, and carbamate insecticides. Further substrate search for CYP6P8 may help to learn about its overexpression in pyrethroid-resistant mosquito. Together with knowledge obtained from enzymatic properties of *An. minimus* CYPOR, this could improve our understanding of P450-mediated detoxification of insecticides, as well as provide a foundation for rational design of P450 synergists specific for P450-mediated pesticide resistance and thus resistant management in mosquito vector control program.

4. Acknowledgment

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5. References

Acevedo, G.R.; Zapater, M. & Toloza, A.C. (2009). Insecticide resistance of house fly, *Musca domestica* (L.) from Argentina. *Parasitology Research*, Vol. 105, No. 2, pp. 489-493, ISSN 0932-0113

Awolola, T.S.; Brooke, B.D.; Hunt, R.H. & Coetzee, M. (2002). Resistance of the malaria vector *Anopheles gambiae s.s.* to pyrethroid insecticides, in south-western Nigeria. *Annals of Tropical Medicine and Parasitology,* Vol. 96, No. 8, pp. 849-852, ISSN 003-4983

Bautista, Ma.A.M.; Miyata, T.; Miura, K. & Tanaka, T. (2009). RNA interference-mediated knockdown of a cytochrome P450, CYP6BG1, from the diamondback moth *Plutella xylostella*, reduces larval resistance to permethrin. *Insect Biochemistry and Molecular Biology*, Vol. 39, No. 1, pp. 38-46, ISSN 0965-1748

Bautista, Ma.A.M; Tanaka, T. & Miyata, T. (2007). Identification of permethrin-inducible cytochrome P450s from the diamondback moth, *Plutella xylostella* (L.) and the possibility of involvement in permethrin resistance. *Pesticide Biochemistry and Physiology,* Vol. 87, No. 1, pp. 85-93, ISSN 0048-3575

Boonsuepsakul, S. (2008). Characterization of CYP6AA3 in baculovirus expression system. Ph.D. Thesis, Mahidol University, Bangkok, Thailand

Boonsuepsakul, S.; Luepromchai, E. & Rongnoparut, P. (2008). Characterization of *Anopheles minimus* CYP6AA3 expressed in a recombinant baculovirus system. *Archives of Insect Biochemistry and Physiology*, Vol. 63, No. 1, pp. 13-21, ISSN 0739-4462

Chareonviriyaphap, T.; Rongnoparut, P. & Juntarumporn, P. (2002). Selection for pyrethroid resistance in a colony of *Anopheles minimus* species A, a malaria vector in Thailand. *Journal of Vector Ecology,* Vol. 27, No. 2, pp. 222-229, ISSN 1081-1710

Chareonviriyaphap, T.; Rongnoparut, P.; Chantarumporn, P. & Bangs, M.J. (2003). Biochemical detection of pyrethroid resistance mechanisms in *Anopheles minimus* in Thailand. *Journal of Vector Ecology,* Vol. 28, No. 1, pp. 108-116, ISSN 1081-1710

Cochran, D.G. (1989). Monitoring for insecticide resistance in field-collected strains of the German cockroach (Dictyoptera: Blattellidae). *Journal of Economic Entomology,* Vol. 82, No. 2, pp. 336-341, ISSN 0022-0493

Cox, C. (2002). Piperonyl butoxide. *Journal of Pesticide Reform,* Vol. 22, pp. 12–20, ISSN 0893-357X

Daware, M.B.; Mujumdar, A.M. & Ghaskadbi, S. (2000). Reproductive toxicity of piperine in Swiss Albino mice. *Planta Medica,* Vol. 66, No. 3, pp. 231-236, ISSN 0032-0943

Djouaka, R.F.; Bakare, A.A.; Coulibaly, O.N.; Akogbeto, M.C.; Ranson, H.; Hemingway, J. & Strode, C. (2008). Expression of the cytochrome P450s, CYP6P3 and CYP6M2 are significantly elevated in multiple pyrethroid resistant populations of *Anopheles gambiae s.s.* from Southern Benin and Nigeria. *BMC genomics,* Vol. 9, p. 538, ISSN 1471-2164

Döhr, O.; Paine, M.J., Friedberg, T.; Robert, G.C.K. & Wolf, R. (2001). Engineering of a functional human NADH-dependent cytochrome P450 system *Proceedings of the National Academy of Sciences of the United States of America,* Vol. 98, No.1, pp. 81-86, ISSN 0027-8424

Duangkaew, P.; Kaewpa, D. & Rongnoparut, P. (2011a). Protective efficacy of *Anopheles minimus* CYP6P7 and CYP6AA3 against cytotoxicity of pyrethroid insecticides in *Spodoptera frugiperda* (*Sf*9) insect cells. *Tropical Biomedicine*, Vol. 28, No. 2, pp. 293-301, ISSN 0127-5720

Duangkaew, P.; Pethuan, S.; Kaewpa, D.; Boonseupsakul, S.; Sarapusit, S. & Rongnoparut, P. (2011b). Characterization of mosquito CYP6P7 and CYP6AA3: Differences in substrate preferences and kinetic properties. *Archives of Insect Biochemistry and Physiology*, Vol. 76, No. 4, pp. 236-248, ISSN 0739-4462

Fakoorziba, M.R.; Eghbal, F. & Vijayan, V.A. (2009). Synergist efficacy of piperonyl butoxide with deltamethrin as pyrethroid insecticide on *Culex tritaeniorhynchus* (Diptera: Culicidae) and other mosquitoe species. *Environmental Toxicology,* Vol. 24, No. 1, pp. 19-24, ISSN 1520-4081

Feyereisen, R. (1999). Insect P450 enzymes. *Annual Review of Entomology,* Vol. 44, pp. 507-533, ISSN 0066-4170

Fornelli, F.; Minervini, F. & Logrieco, A. (2004). Cytotoxicity of fungal metabolites to lepidopteran (*Spodoptera frugiperda*) cell line (*Sf*-9). *Journal of Invertebrate Pathology,* Vol. 85, No. 2, pp. 74-79, ISSN 0022-2011

Gassner, B., Wüthrich, A., Scholtysik, G. & Solioz, M. (1997). The pyrethroids permethrin and cyhalothrin are potent inhibitors of the mitochondrial complex I. *Journal of Pharmacology and Experimental Therapeutics,* Vol. 281, No. 2, pp. 855–860, ISSN 0022-3565

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 Metabolism and Disposition,* Vol. 35, No. 9, pp. 1664–1671, ISSN 0090-9556

Grant, D.F.; Greene, J.F.; Pinot, F.; Borhan, B.; Moghaddam, M.F.; Hummock, B.D.; McCutchen, B.; Ohkawa, H.; Luo, G. & Guenthner, T.M. (1996). Development of an *in situ* toxicity assay system using recombinant baculoviruses. *Biochemical Pharmacology,* Vol. 51, No. 4, pp. 503–515, ISSN 0006-2952

Greene, J.F.; Zheng, J.; Grant, D.F. & Hammock, B.D. (2000). Cytotoxicity of 1,2-epoxynaphthalene is correlated with protein binding and *in situ* glutathione depletion in cytochrome P4501A1 expressing *Sf*-21 cells. *Toxicological Sciences,* Vol. 53, No. 2, pp. 352-360, ISSN 1096-6080

Hargreaves, K.; Koekemoer, L.L.; Brooke, B.D.; Hunt, R.H.; Mthembu, J. & Coetzee, M. (2000). *Anopheles funestus* resistant to pyrethroid insecticides in South Africa. *Medical and Veterinary Entomology,* Vol. 14, No. 2, pp. 181-189, ISSN 0269-283X

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, pp. 653-665, ISSN 0965-1748

Kaltenegger, E.; Brema, B.; Mereiter, K.; Kalchhauser, H.; Kählig, H.; Hofer, O.; Vajrodaya, S. & Greger, H. (2003). Insecticidal pyrido[1,2-a]azepine alkaloids and related derivatives from *Stemona* species. *Phytochemistry,* Vol. 63, No. 7, pp. 803-816, ISSN 0031-9422

Jayaprakasha, G.K.; Singh, R.P.; Pereira, J. & Sakariah, K.K. (1997). Limonoids from *Citrus reticulata* and their moult inhibiting activity in mosquito *Culex quinquefasciatus* larvae. *Phytochemistry,* Vol. 44, No. 5, pp. 843-846, ISSN 0031-9422

Jirakanjanakit, N.; Rongnoparut, P.; Saengtharatip, S.; Chareonviriyaphap, T.; Duchon, S.; Bellec, C. & Yoksan, S. (2007). Insecticide susceptible/resistance status in *Aedes (Stegomyia) aegypti and Aedes (Stegomyia) albopictus* (Diptera: Culicidae) in Thailand during 2003-2005. *Journal of Economic Entomology,* Vol. 100, No. 2, pp. 545-550, ISSN 0022-0493

Kaewpa, D.; Boonsuepsakul, S. & Rongnoparut, P. (2007). Functional expression of mosquito NADPH-cytochrome P450 reductase in *Escherichia coli*. *Journal of Economic Entomology,* Vol. 100, No. 3, pp. 946-953, ISSN 0022-0493

Kumar, S.; Thomas, A.; Sahgal, A.; Verma, A.; Samuel, T. & Pillai, M.K.K. (2002). Effect of the synergist, piperonyl butoxide, on the development of deltamethrin resistance in yellow fever mosquito, *Aedes aegypti L.* (Diptera: Culicidae). *Archives of Insect Biochemistry and Physiology,* Vol. 50, No. 1, pp. 1-8, ISSN 0739-4462

Lertkiatmongkol, P.; Jenwitheesuk, E. & Rongnoparut, P. (2011). Homology modeling of mosquito cytochrome P450 enzymes involved in pyrethroid metabolism: insights into differences in substrate selectivity. *BMC Research Notes*, Vol. 4, p. 321, ISSN 1756-0500

Lian, L.Y.; Widdowson, P.; McLaughlin, L.A. & Paine, M.J. (2011). Biochemical comparison of *Anopheles gambiae* and Human NADPH P450 reductases reveals different 2′-5′-ADP and FMN binding traits. *Plos one,* Vol. 6, No. 5, pp. e20574, ISSN 1932-6203

Liu, N. & Scott, J.G. (1998). Increased transcription of CYP6D1 causes cytochrome P450-mediated insecticide resistance in house fly. *Insect Biochemistry and Molecular Biology,* Vol. 28, No.8, pp. 531-535, ISSN 0965-1748

Maran, E.; Fernández-Franźon, M.; Font, G. & Ruiz, M.J. (2010). Effects of aldicarb and propoxur on cytotoxicity and lipid peroxidation in CHO-K1 cells. *Food and Chemical Toxicology,* Vol. 48, No. 6, pp. 1592–1596, ISSN 0278-6915

Mclaughlin, L.A.; Niazi, U.; Bibby, J.; David, J.-P.; Vontas, J.; Hemingway, J.; Ranson, H.; Sutcliffe, M.J. & Paine M.J.I. (2008). Characterization of inhibitors and substrates of *Anopheles gambiae* CYP6Z2. *Insect Molecular Biology,* Vol. 17, No. 2, pp. 125-135, ISSN 0962-1075

Müller, P.; Donnelly, M.J. & Ranson H. (2007). Transcription profiling of a recently colonized pyrethroid resistant *Anopheles gambiae* strain from Ghana. *BMC Genomics,* Vol. 8, p. 36, ISSN 1471-2164

Müller, P.; Warr, E.; Stevenson, B.J.; Pignatelli, P.M.; Morgan, J.C.; Steven, A.; Yawson, A.E.; Mitchell, S.N.; Ranson, H.; Hemingway, J.; Paine, M.J.I. & Donnelly, M.J. (2008). Field-caught permethrin-resistant *Anopheles gambiae* overexpress CYP6P3, a P450 that metabolises pyrethroids. *PLoS Genetics,* Vol. 4, No. 11, pp. e1000286, ISSN 1553-7390

Murataliev, M..B.; Feyereisen, R. & Walker, F.A. (2004). Electron transfer by diflavin reductases. *Biochimica et Biophysica Acta*, Vol. 1698, No. 1, pp. 1 – 26, ISSN 0006-3002

Naravaneni, R. & Jamil, K. (2005). Evaluation of cytogenetic effects of lambda-cyhalothrin on human lymphocytes. *Journal of Biochemical and Molecular Toxicology,* Vol. 19, No. 5, pp. 304-310, ISSN 1095-6670

Nelson, D.R.; Koymans, L.; Kamataki, T.; Stegeman, J.J.; Feyereisen, R.; Waxman, D.J.; Waterman, M.R.; Gotoh, O.; Coon, M.J; Estabrook, R.W.; Gunsalus, I.C. & Nebert, D.W. (1996). P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics,* Vol. 6, No. 1, pp. 1-42, ISSN 0960-314X

Okamiya, H.; Mitsumori, K.; Onodera, H.; Ito, S.; Imazawa, T.; Yasuhara, K. & Takahashi, M. (1998). Mechanistic study on liver tumor promoting effects of piperonyl

butoxide in rats. *Archives of Toxicology,* Vol. 72, No. 11, pp. 744–750, ISSN 0340-5761

Omura, T. & Sato, R. (1964). The carbon monoxide-biding pigment of liver microsome I. Evidence for its hemoprotein nature. *The Journal of Biological Chemistry,* Vol. 239, pp. 2370-2378, ISSN 0021-9258

Ortiz de Montellano, P.R. (2005). *Cytochrome P450: structure, mechanism, and biochemistry*, Kluwer Academic/Plenum Piblishers, ISBN 0-306-48324-6, New York, USA.

Patel, S.; Bajpayee, M.; Pandey, A.K.; Parmar, D. & Dhawan, A. (2007). *In vitro* induction of cytotoxicity and DNA strand breaks in CHO cells exposed to cypermethrin, pendimethalin and dichlorvos. *Toxicology In Vitro,* Vol. 21, No. 8, pp. 1409–1418, ISSN 0887-2333

Price, N.R. (1991). Insect resistance to insecticides: mechanisms and diagnosis. *Comparative Biochemistry and Physiology - C Pharmacology Toxicology and Endocrinology,* Vol. 100, No. 3, pp. 319-326, ISSN 0742-8413

Ranasinghe, C. & Hobbs, A.A. (1998). Isolation and characterization of two cytochrome P450 cDNA clones for CYP6B6 and CYP6B7 from *Helicoverpa armigera* (Hubner): possible involvement of CYP6B7 in pyrethroid resistance. *Insect Biochemistry and Molecular Biology,* Vol. 28, No. 8, pp. 571-580, ISSN 0965-1748

Rodpradit, P.; Boonsuepsakul, S.; Chareonviriyaphap, T.; Bangs, M.J. & Rongnoparut, P. (2005). Cytochrome P450 genes: molecular cloning and overexpression in a pyrethroid-resistant strain of *Anopheles minimus* mosquito. *Journal of the American Mosquito Control Association,* Vol. 21, No. 1, pp. 71-79, ISSN 8756-971X

Rongnoparut, P.; Boonsuepsakul, S.; Chareonviriyaphap, T. & Thanomsing, N. (2003). Cloning of cytochrome P450, CYP6P5, and CYP6AA2 from *Anopheles minimus* resistant to deltamethrin. *Journal of Vector Ecology,* Vol. 28, No. 2, pp. 150-158, ISSN 1081-1710

Saito, S.; Sakamoto, N. & Umeda, K. (2005). Effects of pyridalyl, a novel insecticidal agent, on cultured *Sf*9 cells. *Journal of Pesticide Science,* Vol. 30, No. 1, pp. 17-21, ISSN 0385-1559

Sarapusit, S. (2009). Study of NADPH-cytochrome P450 oxidoreductase from *Anopheles minimus* mosquito. Ph.D Thesis, Mahidol University, Bangkok, Thailand

Sarapusit, S.; Xia, C.; Misra, I.; Rongnoparut, P. & Kim, J-JP. (2008). NADPH-Cytochrome P450 oxidoreductase from *Anopheles minimus* mosquito: Kinetic studies and the influence of Leu86 and Leu219 for cofactors binding and protein stability. *Archives of Biochemistry and Biophysics,* Vol. 477, No. 1, pp. 53-59, ISSN 0739-4462

Sarapusit, S.; Pethuan, S. & Rongnoparut, P. (2010). Mosquito NADPH-cytochrome P450 oxidoreductase: kinetics and role of phenylalanine amino acid substitutions at Leu86 and Leu219 in CYP6AA3-mediated deltamethrin metabolism. *Archives of Insect Biochemistry and Physiology*, Vol. 73, No. 4, pp. 232-244, ISSN 0739-4462

Schmuck, G. & Mihail, F. (2004). Effects of the carbamates fenoxycarb, propamocarb and propoxur on energy supply, glucose utilization and SH-groups in neurons. *Archives*

of Toxicology, Vol. 78, No. 6, pp. 330–337, Epub 2004 Feb 19, ISSN 0340-5761

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 Metabolism and Disposition,* Vol. 37, No. 1, pp. 221–228, ISSN 0090-9556

Sem, D.S & Kasper, C.B. (1994). Kinetic mechanism for the model reaction of NADPH-cytochrome P450 oxidoreductase with cytochrome c. *Biochemistry*, Vol. 33, No. 4, pp. 12012–12021, ISSN 0006-2960

Sem, D.S. & Kasper, C.B. (1995). Effect of ionic strength on the kinetic mechanism and relative rate limitation of steps in the model NADPH-cytochrome P450 oxidoreductase reaction with cytochrome c. *Biochemistry*, Vol. 34, No. 39, pp.12768–12774, ISSN 0006-2960

Shen, A.L., Porter, T.D., Wilson, T.E., Kasper, C.B. (1989) Structural analysis of the FMN binding domain of NADPH-cytochrome P-450 oxidoreductase by site-directed mutagenesis. *Journal of Biological Chemistry*, Vol. 254, No. 13, pp. 7584–7589, ISSN 0021-9258

Shono, T.; Ohsawa, K. & Casida, J.E. (1979). Metabolism of *trans*- and *cis*-permethrin, *trans*- and *cis*-cypermethrin and decamethrin by microsomal enzymes. *Journal of Agriculture and Food Chemistry,* Vol. 27, No. 2, pp. 316-325, ISSN 0021-8561

Stevenson, B.J.; Bibby, J.; Pignatelli, P.; Muangnoicharoen, S.; O'Neill, P.M.; Lian, L-Y; Müller, P.; Nikou, D.; Steven, A.; Hemingway, J.; Sutcliffe, M.J. & Paine, M.J.I. (2011). Cytochrome P450 6M2 from the malaria vector *Anopheles gambiae* metabolizes pyrethroids: Sequential metabolism of deltamethrin revealed. *Insect Biochemistry and Molecular Biology,* Vol. 41, No. 7, pp. 492-502, ISSN 0965-1748

Tomita, T.; Liu, N.; Smith, F.F.; Sridhar, P. & Scott, J.G. (1995). Molecular mechanisms involved in increased expression of a cytochrome P450 responsible for pyrethroid resistance in the housefly, *Musca domestica*. *Insect Molocular Biology,* Vol. 4, No. 3, pp. 135-140, ISSN 0962-1075

Vijayan, V.A.; Sathish Kumar, B.Y.; Ganesh, K.N.; Urmila, J.; Fakoorziba, M.R. & Makkapati, A.K. (2007). Efficacy of piperonyl butoxide (PBO) as a synergist with deltamethrin on five species of mosquitoes. *The Journal of Communicable Diseases,* Vol. 39, No. 3, pp. 159-163, ISSN 0019-5638

Wang, M.; Roberts, D.L.; Paschke, R.; Shea, T.M.; Masters, B.S. & Kim, J.J. (1997). Three-dimensional structure of NADPH-cytochrome P450 reductase: prototype for FMN- and FAD-containing enzymes. *Proceedings of the National Academy of Sciences of the United States of America,* Vol. 94, No. 16, pp. 8411-8416, ISSN 0027-8424

Wondji, C.S.; Morgan, J.; Coetzee, M.; Hunt, R.H.; Steen, K.; Black IV, W.C.; Hemingway, J. & Ranson, H. (2007). Mapping a Quantitative Trait Locus (QTL) conferring pyrethroid resistance in the African malaria vector *Anopheles funetus*. *BMC Genomics*, Vol. 8, p. 34, ISSN 1471-2164

Yaicharoen, R.; Kiatfuengfoo, R; Chareonviriyaphap, T. & Rongnoparut, P. (2005). Characterization of deltamethrin resistance in field populations of *Aedes aegypti* in Thailand. *Journal of Vector Ecology,* Vol. 30, No. 1, pp. 144-150, ISSN 1081-1710

Zhang, M. & Scott, J.G. (1996). Cytochrome b5 is essential for cytochrome P450 6D1-mediated cypermethrin resistance in LPR house flies. *Pesticide Biochemistry and Physiology,* Vol. 55, No. 2, pp. 150-156, ISSN 0048-3575

Zhu, F.; Parthasarathy, R.; Bai, H.; Woithe, K.; Kaussmann, M.; Nauen, R.; Harrison, D.A. & Palli S.R. (2010). A brain-specific cytochrome P450 responsible for the majority of deltamethrin resistance in the QTC279 strain of *Tribolium castaneum*. *Proceedings of the National Academy of Sciences of the United States of America,* Vol. 107, No. 19, pp. 8557-8562, ISSN 0027-8424

Part 3

Pest Management

13

Bioactive Natural Products from Sapindaceae Deterrent and Toxic Metabolites Against Insects

Martina Díaz and Carmen Rossini
Laboratorio de Ecología Química, Facultad de Química, Universidad de la República, Uruguay

1. Introduction

The Sapindaceae (soapberry family) is a family of flowering plants with about 2000 species occurring from temperate to tropical regions throughout the world. Members of this family have been widely studied for their pharmacological activities; being *Paullinia* and *Dodonaea* good examples of genera containing species with these properties. Besides, the family includes many species with economically valuable tropical fruits, and wood (Rodriguez 1958), as well as many genera with reported anti-insect activity.

Antioxidant, anti-inflammatory and anti-diabetic properties are the pharmacological activities most commonly described for this family (Sofidiya et al. 2008; Simpson et al. 2010; Veeramani et al. 2010; Muthukumran et al. 2011). These activities are in some cases accounted for isolated phenolic compounds such as prenylated flavonoids (Niu et al. 2010), but in many cases, it is still unknown which are the active principles. Indeed, there are many studies of complex mixtures (crude aqueous or ethanolic extracts) from different species in which several other pharmacological activities have been described without characterization of the active compounds [*e.g.* antimigrane (Arulmozhi et al. 2005), anti-ulcerogenic (Dharmani et al. 2005), antimalarial (Waako et al. 2005), anti-microbial (Getie et al. 2003)].

Phytochemical studies on Sapindaceae species are abundant and various kinds of natural products have been isolated and elucidated. Examples of these are flavonoids from *Dodonaea* spp. (Getie et al. 2002; Wollenweber & Roitman 2007) and *Koelreuteria* spp. (Mahmoud et al. 2001), linear triterpenes from *Cupaniopsis* spp. (Bousserouel et al. 2005) and caffeine, xanthenes and cathequines from *Paullinia* spp. (Benlekehal et al. 2001; Sousa et al. 2009). All these compounds are naturally occurring in almost every plant family, however, the Sapindaceae do produce an unusual group of secondary metabolites: the cyanolipids (Avato et al. 2005). Eventhough these compounds exhibit a potential health hazard for humans and animals, for the plants, cyanolipids may have a protective physiological role. However, not many investigations have been developed involving the study of the ecological interactions among the plants producing them and other sympatric organisms. On the other hand, the toxicity of these cyanocompounds might be a potential source of pesticides. Indeed, not only cyanocompounds, but also a wide range of species of Sapindaceae have been tested on their anti-insect activity. Several extracts, fractions or pure compounds of different phenological stages, have been tested against diverse species of lepidopterans, dipterans and coleopterans of major importance in agriculture as well as in veterinary and medical applications. Examples of this include the larvicidal activity of *Magonia pubescens* against *Aedes aegypti*

(Diptera: Culicidae) (Arruda et al. 2003a,b); the toxicity of *Sapindus* spp. against *Sitophilus oryzae* (Coleoptera: Curculionidae) (Zidan et al. 1993; Rahman et al. 2007); and the toxicity of *Dodonaea* spp. against *Spodoptera* spp. (Lepidoptera: Noctuidae) (Abdel et al. 1995; El-Din & El-Gengaihi 2000; Deepa & Remadevi 2007; Malarvannan et al. 2008). The activity against different pest models of extracts from various South American species within the Sapindaceae was recently described. These species included *Allophylus edulis*, *Dodonaea viscosa* and *Serjania meridionalis*, from which isolated metabolites with anti-insect capacities have not yet been described (Castillo et al. 2009). This chapter will examine the available information on extracts and secondary metabolites from Sapindaceae focused on their defensive role for the plant against herbivory; and consequently this appraisal will also present a compilation of potential anti-insect agents from Sapindaceae.

2. Sapindaceae: its anti-insect potential

Anti-insect activity has been described in at least 15 of the 202 genera (Anonymous 2011) belonging to this family. Among these findings, the cases in which the bioactive compounds were isolated represent the least. It has been in general tested the activity of aqueous or ethanolic extracts from different organs and from plants of different phenological stages against a variety of insect targets using different bioassay designs. As a consequence, different modes of action have been described. Extracts have revealed to be potentially deterrent agents, growth inhibitors and even toxic agents against different genera of insects. The following appraisal comprises the main Sapindaceae genera from which extracts or isolated compounds with anti-insect activity have been described.

2.1 *Sapindus*

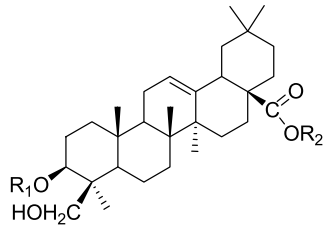
A vast number of species showing great potential as anti-insect agents belong to this genus. *Sapindus saponaria*, a tree widely distributed in Central and South America, is also frequently used as ornamental (Lorenzi 2004). Brazilian people commonly prepare homemade soap from this tree; and use its seeds to make handcrafts. Its wood is broadly used in construction. Its fruits and roots are popularly used as painkillers, astringents, expectorants and diuretics (Ferreira Barreto et al. 2006). Besides, its medicinal potential as healing and anti thrombotic agents has been studied. Research on that area has revealed that flavonoids in the leaf extracts are responsible for those activities (Meyer Albiero et al. 2002). On the other hand, much research has been devoted to the anti-insect capacity of extracts from different organs of this plant. Boiça Junior et al (2005), on their search for activity against larvae of the cabbage pest *Plutella xylostella* (Lepidoptera: Plutellidae) investigated eighteen plant species from a variety of families, finding that aqueous leaf extract of *S. saponaria* was one of the most active products. The extract produced 100 % of mortality in tests where the larvae were offered cabbage foliage disks coated with the extracts to be evaluated as a sole food (Boiça Junior et al. 2005). In another study, the aqueous fruit extract of this tree showed deterrent properties against another cabbage pest, *Ascia monuste orseis* (Lepidoptera: Pieridae). In this case the activity was comparable to that showed by aqueous extracts of the neem tree, *Azadirachta indica*, the newest botanical pesticide in the market (Isman et al. 1996; Medeiros et al. 2007). The aqueous seeds´ extracts were evaluated against another lepidopteran, *Spodoptera frugiperda* (Lepidoptera: Noctuidae), showing strong effect on larvae development and midgut trypsin activity (dos Santos et al. 2008). Not only against lepidopterans has this tree revealed anti-insect potential, but also against other insect orders.

For instance, a saponin extract from fruits from this species showed toxicity against adults of the greenhouse whitefly *Trialeurodes vaporariorum* (Hemiptera: Aleyrodidae) (Porras & Lopez-Avila 2009); and complete ethanolic extracts from fruits have shown larvicidal and morphological alterations effects on the mosquito *Aedes aegypti* (Diptera: Culicidae) (Ferreira Barreto et al. 2006). Some other saponins presenting other kinds of biological activity, isolated from the fruits of this species, are shown in Figure 1 (Lemos et al. 1992; Ribeiro et al. 1995).

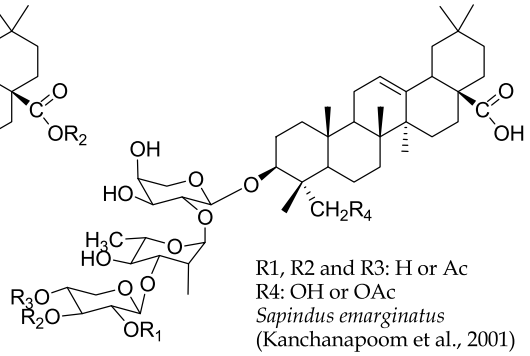
Sapindus emarginatus, another tree from this genus, widely distributed in India, has also demonstrated larvicidal activity of its fruit extract against three important vector mosquitoes: *A. aegypti*, *Anopheles stephensi* and *Culex quimquefasciatus* (Diptera: Culicidae) (Koodalingam et al. 2009). Later, this group has also investigated the impact of the extracts on the activity of mosquito phosphatases and esterases to gain an insight into the extent of disturbance in metabolic homeostasis inflicted upon exposure to the extract (Koodalingam et al. 2011). Previous reports on this species have shown that the pericarps contain triterpene saponins (Figure 1), which are commonly used as antifertility, antipruritic and anti-inflammatory agents in traditional Indian and Thai medicine (Jain 1976; Kanchanapoom et al. 2001). Perhaps, anti insect activity may be due to saponins in this plant similarly to the case of *Sapindus saponaria* (Porras et al. 2009).

Activity of members of this genus against other insect orders, further than dipterans and lepidopterans, has also been evaluated, including coleoptera and lice. That is the case of the extract from *Sapindus trifoliatus* fruit cortex which showed activity against the red flour beetle *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) (Mukherjee & Joseph 2000). In this case, weight gain was significantly reduced when larvae were fed on diets including the extract at different doses; and females topically treated -upon emergence- with the extract laid fewer viable eggs. (Mukherjee & Joseph 2001). Another ethanolic fruit extracts, in this case from *Sapindus mukorossi*, also showed anti coleopteran activity against another pest of stored grains, *Sitophilus oryzae* (Coleoptera: Curculionidae) and also against *Pediculus humanus* (Phthiraptera: Pediculidae) (Rahman et al. 2007). Finally, from the methanolic extract of fruits of this species triterpenic saponins (Figure 1) have also been isolated and these natural products demonstrated their potential as growth regulators and antifeedants against *Spodoptera littura* (Lepidoptera: Noctuidae), both as glycosides and as free genines (Saha et al. 2009). In this particular study, it was verified that upon hydrolysis of the saponins, the growth regulatory activity was improved, whereas very little difference was found in regard to the antifeedant activity.

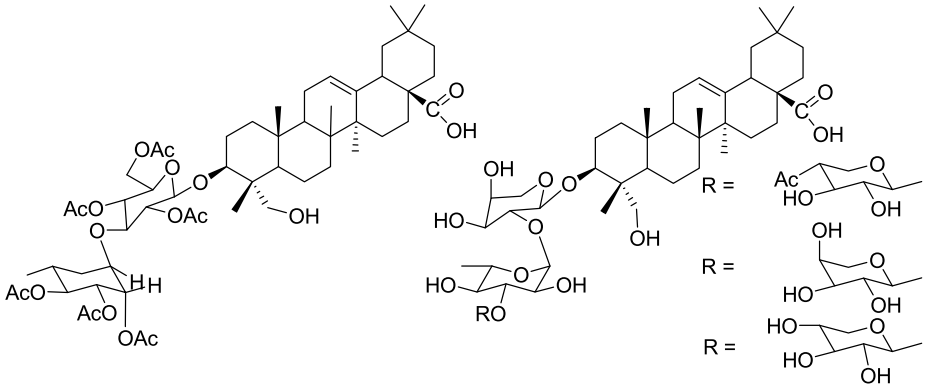
All in all, the genus *Sapindus* contains a variety of species which have been studied on their activity for some insects from different orders. In spite of the fact that not many reports do exist on the action of isolated compounds, the previous ethnobotanical uses of *Sapindus* spp. and the isolation of some active saponins from this genus, may suggest that this group of secondary metabolites might be related to the anti-insect activity. Saponins -glycosides of sapogenins containing a monosaccharide or a polysaccharide unit- reduce the surface tension becoming biological detergents. They are widely distributed secondary plant metabolites, found among almost 100 plant families (Bruneton 1995). Being effective defences for some insects (Plasman et al. 2001; Prieto et al. 2007), saponins have been implied in mechanisms of plant resistance against potential herbivores (Nielsen et al. 2010). The genus *Sapindus*, rich in this kind of compounds, may therefore be promissory raw material to develop plant pest control products. Further information can be found at recent works reviewing saponins from *Sapindus* spp. and their activity (Pelegrini et al. 2008; Sharma et al. 2011).



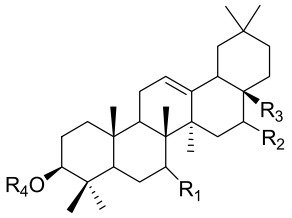
R1: Xyl (OAc)-Ara-Rha
R2: Glc-Glc-Rha
Sapindus mukorossi
(Saha et al., 2010)



R1, R2 and R3: H or Ac
R4: OH or OAc
Sapindus emarginatus
(Kanchanapoom et al., 2001)



Sapindus saponaria (Ribeiro et al, 1995; Lemos et al, 1992)



	R1	R2	R3	R4
1	H	H	CH_2OH	-Xyl(2→1)Glc, 3→1 Ara
2	H	H	CH_2OH	-Glc(2→1)Glc, 3→1 Ara
3	H	H	CH_2OH	-Xyl(3→1)Glc(6→1)-Ac
4	α-OCH_3	H	CH_2OH	-Glc(2→1)Glc, 3→1 Ara
5	H	β-OH	CH_3	-Xyl(2→1)Glc, 3→1 Ara
6	H	β-OH	CH_3	-Glc(2→1)Glc, 3→1 Ara

Nepheliosides **1-6** *Nephelium lappaceum* (Ito et al., 2003)

Fig. 1. Saponins isolated from *Sapindus* and *Nephelium* spp.

2.2 *Dodonaea*

From this genus, there are two species to which almost all research has been devoted: *Dodonaea angustifolia* and *Dodonaea viscosa*. These two species are considered by some taxonomists to be synonymous, while others recognize *D. angustifolia* as a sub-species of *D. viscosa* (cited in Omosa et al. 2010). *D. angustifolia* is widely distributed in Australia, Africa, Asia and South America; and it has been employed until present days in traditional medicine all over the world. It is traditionally used as analgesic, laxative, antipyretic, and to treat rheumatism, eczema, and skin ailments (Malarvannan et al. 2009; Omosa et al. 2010). *Dodonaea viscosa* is a shrub, rarely a small tree, widely distributed in tropical and subtropical areas of both hemispheres. It is used in folk medicine as a febrifuge, a diaphoretic drug, and also for the treatment of rheumatism, gout, inflammations, swelling and pain (Niu et al. 2010).

Anti-insect activity has been described for extracts from both plant species mostly against lepidopterans (Malarvannan & Subashini 2007; Malarvannan et al. 2008; 2009; Sharaby et al. 2009). For instance, extracts of leaves of *D. angustifolia* (obtained with hexane, petroleum ether, chloroform, acetone and water) were tested in field bioassays, showing to be effective biocontrol agents for the larvae of *Earias vitella* (Lepidoptera: Noctuidae) (Malarvannan et al. 2007). Besides, those extracts also showed ovicidal activity against *Helicoverpa armigera* (Lepidoptera: Noctuidae) (Malarvanan 2003). However, while in the case of extracts from different organs (fruits, leaves and twigs) of *D. viscosa* coming from Uruguay, none of these products proved to be active against the polyphagous *Spodoptera littoralis* (Lepidoptera: Noctuidae) (Castillo et al. 2009), insects from other orders were deterred by *D. viscosa* extracts. Interestingly, while extracts from leaves and twigs exhibited good activity against aphids (*Rhopalosiphum padi* and *Myzus persicae*) and a coleopteran (*Epilachna paenulata*), they were innocuous to beneficial insects (*Apis mellifera*) (Castillo et al. 2009). This selectivity makes *D. viscosa* a good candidate from which to develop botanical pesticides. Another independent study also showed a strong contact activity of the seed extracts against the coleopteran, *S. oryzae* (Zhao et al. 2006).

D. angustifolia is known to contain essential oils, flavonoids, terpenoids, phenols, coumarins, sterols and unidentified alcohols (Malarvannan et al. 2008). Meanwhile several flavonoids, diterpenoid acids and saponins have been isolated from *D. viscosa* (Niu et al. 2010). However, the chemical basis for the pesticide and antifeedant activities remains unclear as tests on individual compounds have not been performed. Nevertheless, a series of clerodane diterpenoids (Figure 2) and prenylated flavonoids (Figure 3) were isolated from the aerial parts of *D. viscosa* from China, having them not shown larvicidal activity against two mosquito species tested (Niu et al. 2010), however the authors stated that previous studies showed activity of these clerodanes against two lepidopterans (*Plutella xylostella* and *Pieris rapae*) and against the coleopteran *Sitophilus oryzae*.

At the same time, an investigation on this family of compounds from *D. angustifolia* from Kenya showed that the extracts from the leaf surface of this plant is composed mainly by clerodanes (Figure 2) and also by methylated flavones and flavonols (Figure 3) (Omosa et al.). Clerodanes isolated from *D. viscosa* and *D. angustifolia* belong to the *neo*-clerodane group. As it is well known, these secondary metabolites have a structure based on the carbon skeleton and absolute stereochemistry of clerodin (Klein Gebbinck et al. 2002) isolated first from *Clerodendron infortunatum* (Lamiaceae) (Banerjee 1936). This large group of plant secondary metabolites have been described mainly from Lamiaceae and Asteraceae, and they have exhibited a wide range of anti-insect properties as it has been reviewed

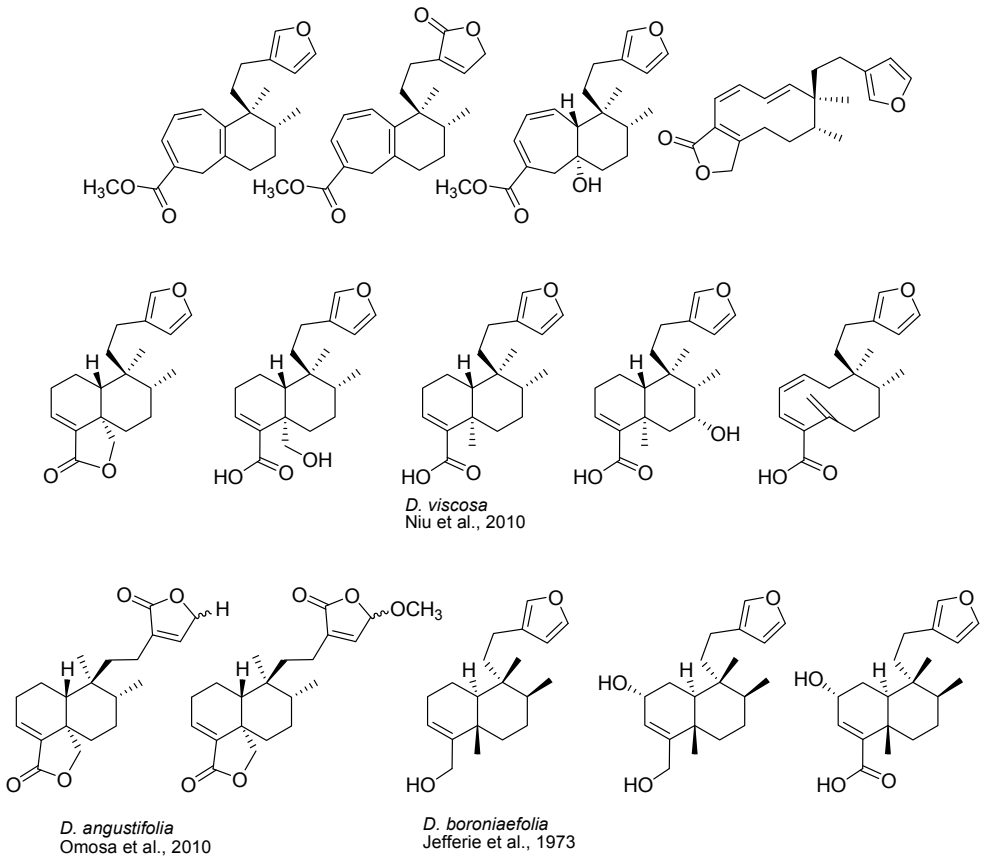


Fig. 2. Clerodanes from *Dodonaea* spp. Structures shown were isolated from *D. viscosa* (Niu et al. 2010) with the exception of the indicated ones.

previously (Klein Gebbinck et al. 2002; Sosa & Tonn 2008). Worth to be noticed, an earlier work by Jefferies et al. (1973) reported the occurrence of various diterpenes in another species, *Dodonaea boroniaefolia*, of the opposite configuration in the main skeleton (Figure 3), that is *ent*-clerodanes.

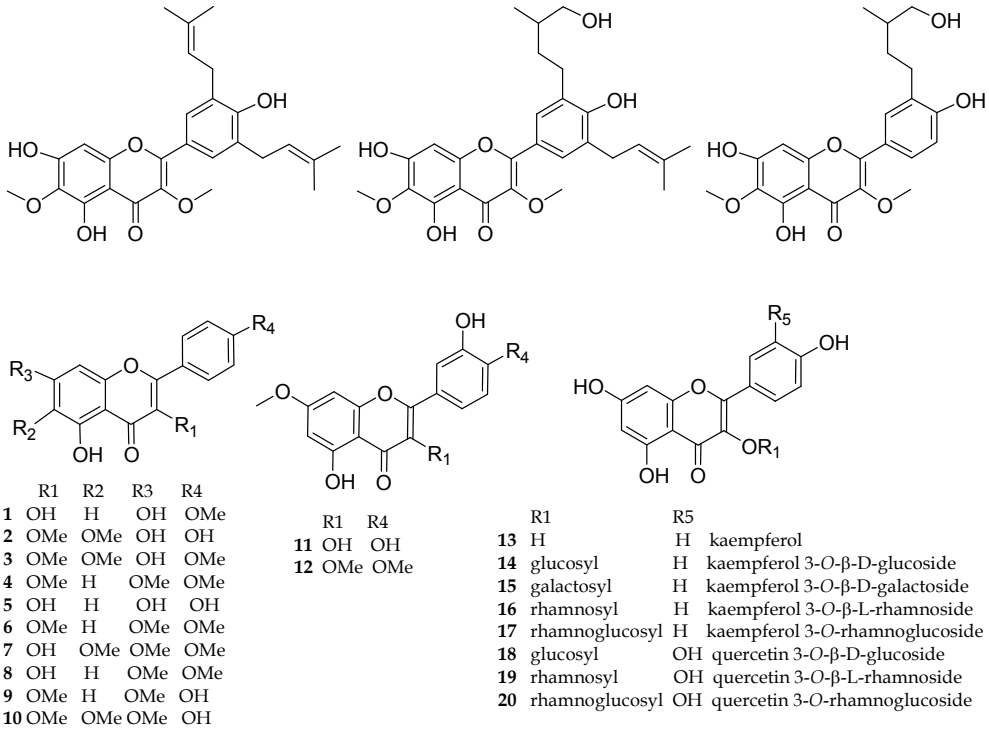


Fig. 3. Flavonoids from *Dodonaea* spp. (Niu et al. 2010; Omosa et al. 2010; Teffo et al. 2010).

2.3 *Magonia and Paullinia species*

Paullinia spp. is one of the few purine alkaloid-containing genera used to prepare stimulant drinks worldwide (Weckerle et al. 2003). *Paullinia clavigera* grows in primary forests and shores of the South American Amazonic aquatic ecosystems, and it has been studied mostly in Perú for the control of different insect pests (cited in Pérez et al. 2010). The toxicity of aqueous extracts of lianas from this species against larvae of *Anopheles benarrochi (*Diptera: Culicidae) (Pérez & Iannacone 2004) and afterwards the mortality and repellence of such extracts against larvae of *Rhynchophorus palmarum* (Coleoptera: Curculionidae) (Pérez & Iannacone 2006) were tested. Besides, the activity of aqueous extracts against *Eupalamides cyparissias* (Lepidoptera: Castniidae), and the activity of hydroalcoholic extracts against *Tuthillia cognata* (Hemiptera: Psyllidae) (Pérez et al. 2008) were also reported, showing the potential of this vegetal species as a biocontrol agent against different insect orders. However, it is still unclear which compounds are responsible for the anti-insect effects of *P. clavigera*. Flavonoids, phenols, triterpenes and saponins were detected in a phytochemical study in extracts from the stem cortex of *P. clavigera.* (Pérez et al. 2010); and other species from the same genus (*P. cururu*) contain saponins, tannins and polyphenols (Wilbert & Haiek 1991).

Magonia pubescens, widely distributed in the Brazilian Cerrado, is commonly used in the construction industry. It has been mostly studied for its larvicidal activity against *A. aegypti* (Arruda et al. 2003; da Silva et al. 2003; Rodrigues et al. 2006). In this case, one of the most active fractions from the ethanolic extract of stem barks was shown to be rich in tannins, and specially in a proanthocyanidin (catequic tannin which structure is shown in Figure 4) (Silva et al. 2004). Tannins are largely distributed in nature, usually being the active principles of plants used in traditional medicine. Condensed tannins have a great ability to interact with metallic ions and macromolecules and to form soluble complexes with electron-donor groups such as the ones found in alkaloids and proteins. That may be one of the reasons explaining their toxicity against different organisms, including insects, fungi and bacteria. Morphological alterations caused by this active fraction on the epithelium of the midgut of larvae of *A. aegypti* resembled the ones recorded for tannic acid (Rey et al. 1999). In a side note, it is worth to notice that this vegetal species has also demonstrated potential on its ethanolic extract of stem barks, as acaricide against the larvae of the common cattle tick, *Rhipicephalus (Boophilus) sanguineus* (Acari: Ixodidae) (Fernandes et al. 2008).

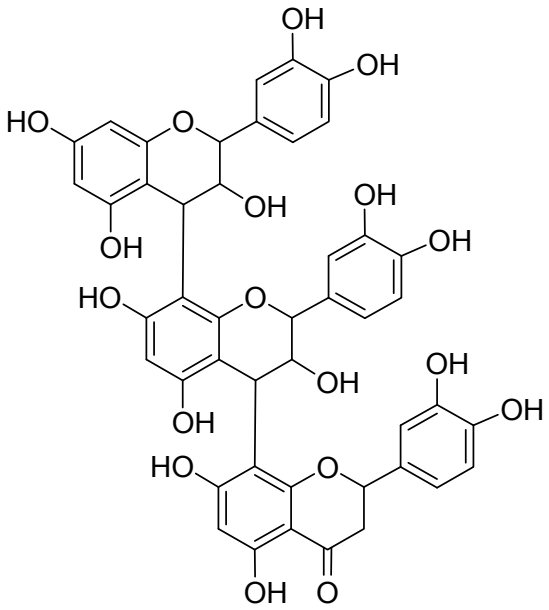


Fig. 4. Catequic tannin with larvicidal activity against *A. aegypti* isolated from *Magonia pubescens* (Silva et al. 2004).

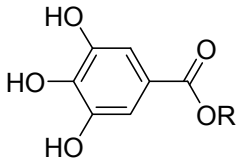
2.4 Miscellaneous

Talisia esculenta, locally known as pitomba occurs in northern and northeastern Brazil. Its fruits are edible to humans and birds which disperse the seeds. However, popular information also mentions that chickens die after ingesting the fruit (cited in Macedo et al.

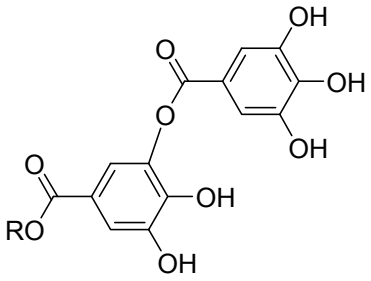
2002). *Koelreuteria paniculata* is popularly grown as an ornamental tree in temperate regions all across the world (Kamala-Kannan et al. 2009). From these two species of Sapindaceae, lectins have been isolated from their seeds (Macedo et al. 2002; Macedo et al. 2003). Lectins from *T. esculenta* inhibited larval growth of two bruchids *(Callobroschus maculatus* and *Zabrotes subfasciatus*) (Macedo et al. 2002). And in the case of *K. paniculata*, lectins not only showed insecticide activity against *C. maculatus* but also against *Anagasta kuehniella* (Lepidoptera: Pyralidae) (Macedo et al. 2003). Plant lectins are a large group of proteins defined as ''plant proteins that possess at least one non-catalytic domain that binds reversibly to a specific mono- or oligosaccharide'' (Peumans & Van Damme 1995). Plant lectines have been implied in many ecological roles, being their action as defences against insects one of the latest described (Murdock & Shade 2002; Van Damme et al. 2008). Their mechanisms of action as anti-insect agents are yet poorly understood, with many emerging hypotheses proposed (Van Damme et al. 2008). According to Macedo et al. (2003), the action of *K. paniculata* lectins on *C. maculatus* and *A. kuehniella* larvae may involve *(1) binding to glycoconjugates on the surface of epithelial cells along the digestive tract, (2) binding to glycosylated digestive enzymes, thereby inhibiting their activity, and (3) binding to the chitin component of the peritrophic membrane (or equivalent structures) in the insect midgut*. Finally, regarding *T. esculenta*, its aqueous seeds extracts were studied on its effect on *S. frugiperda* larvae which development was negatively affected, but the activity of the midgut trypsin was not inhibited (dos Santos et al. 2008).

The red maple, *Acer rubrum*, is another Sapindaceae from which bioactive compounds have been isolated. This prominent maple occurs in hardwood forests, being avoided by several potential sympatric consumers [for instance, larvae of *Malacossoma disstria* (Lepidoptera: Lasiocampidae), and the North American beavers, *Castor canadensis*] (Abou-Zaid et al. 2001). The main constituents of an aqueous leaf extract have been phytochemically characterized as ellagic acid, gallate derivatives (structures **1-7** in Figure 5) and glycosides of flavonoids (quercetin and structures **13-20** in Figure 3). When these compounds were tested by themselves against *M. disstria* larvae, it was found that all gallate derivatives exhibited deterrent activity, but not the flavonoids. Among gallate derivatives, compounds **2** and **4-7** in Figure 5 were the five most active compounds. Perhaps in this case, the feeding deterrence effect of the extracts may be traced to the galloyl moiety in its secondary metabolites.

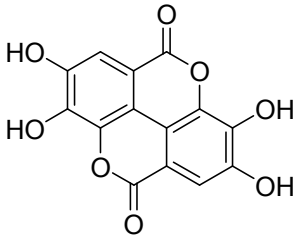
Blighia sapida, commonly known as Ackee, is an evergreen tree, native from West African wild forests. In the late 18th century, the plant was introduced in Jamaica, where nowadays its fruit has been adopted as the national fruit (cited in Gaillard et al. 2011). Its bark is used as fish poison and also in folk medicine in the treatment of malaria, ulcers, back aches and headaches (Kayode 2006). The plant contains triterpenic and steroidal saponins, alkaloids, polyphenols and aminoacidic secondary metabolites (Mazzola et al. 2011). By ingestion, the unripe fruits can cause vomiting and circulatory collapse in humans due to the presence of hypoglycin-A (seeds and flesh) and hypoglycin-B (seeds) (Figure 6) (Hassall et al. 1954; Hassall & Reyle 1955; Gaillard et al. 2011). Acetone and ethanolic extracts of the fruits showed repellent properties against stored-product pests, namely, *C. maculatus*, *Cryptolestes ferrugineus* (Coleoptera: Cucujidae), and *Sitophilus zeamais* (Coleoptera: Curculionidae) (Khan & Gumbs 2003). Furthermore, ethanol, acetone, hexane, methanol, chloroform, and water extracts from the seeds were evaluated on their repellence against *T. castaneum,* demonstrating the aqueous extract to be the most active (Khan et al. 2002).



1 R = H Gallic acid
2 R = Me Methyl gallate
3 R = Et Ethyl gallate
4 R = β-D-Glc 1-*O*-Galloyl-b-D-glucosa
5 R = α-L-Rha 1-*O*-Galloyl-a-L-rhamnose

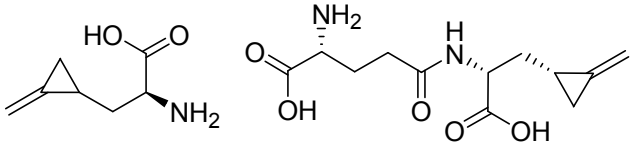


6 R = OH *m*-Digallate
7 R = OC_2O_5 Ethyl *m*-digallate



8 ellagic acid

Fig. 5. Ellagic acid and gallate derivatives isolated from *A. rubrum.* (Abou-Zaid et al. 2001).



Hypoglycin-A Hypoglycin-B

Fig. 6. Hypoglycins isolated from the fruits of *B. sapida.*

Serjania lethalis is another species that has been studied in its anti-insect activity. The ethanolic stem bark extract showed larvicidal activity against *A. aegypti* (Rodrigues et al. 2006). In this study the active compounds were not identified. However, the presence in this species of tannins, flavonoids and of saponins (serjanosides) has been reported (Teixeira et al. 1984; de Sousa Araújo et al. 2008). *S. meridionalis,* a species phytochemically not described, exhibited deterrent activity against *E. paenulata* and *M. persicae* (Hemiptera: Aphididae) when its ethanolic leaf extracts were assayed. Disappointingly, this extract was also toxic against beneficial insects (honey bees) (Castillo et al. 2009).

From the genus *Nephelium*, the species *N. lappaceum* is commonly known for its edible fruit "rambutan". It is native from Southeast Asia where the fruits are an important commercial crop (Palanisamy et al. 2008). These fruits have shown potential on its ethanol seed extract, against *S. oryzae*, revealing to reduce esterase and glutathione-S-transferase activities from such insect (Bullangpoti et al. 2004). *N. maingayi,* native from Malasya and Indonesia, has

also edible fruits and it has not been studied in its anti-insect activity. However, six saponins, namely, nepheliosides **1-6** (Figure 1) were isolated from a chloroform extract of its bark exhibiting cytotoxic activity when evaluated against a panel of human cancer cell lines (Ito et al. 2004).

3. Cyanocompounds

As stated, Sapindaceae are rich –in their seeds- in toxic cyanolipids (Figure 8), *e.g.* fatty acid esters of α- and γ- hydroxynitriles (Mikolajczak 1977; Seigler 1991). Although, some works report the occurrence of cyanolipids also in members of the Hippocastanaceae (Mikolajczak 1977; Bjarnholt & Møller 2008) and the Boraginaceae (Mikolajczak et al. 1969; Seigler et al. 1970), it appears that later investigations have confirmed that these metabolites are characteristic only of the Sapindaceae (Seigler 1976; Avato et al. 2005). The cyanolipids are usually extracted in the seed oils where the amounts vary broadly within the species (Dinesh & Hasan; Selmar et al. 1990; Hasan et al. 1994; Ucciani et al. 1994; Hasan & Roomi 1996; Sarita et al. 2002; Avato et al. 2005; Avato et al. 2006), ranging from only 3% in *Paullinia cupana var. sorbilis* (Guarana) (Avato et al. 2003), to 58% in *Schleichera trijuga* (Mikolajczak & Smith 1971).

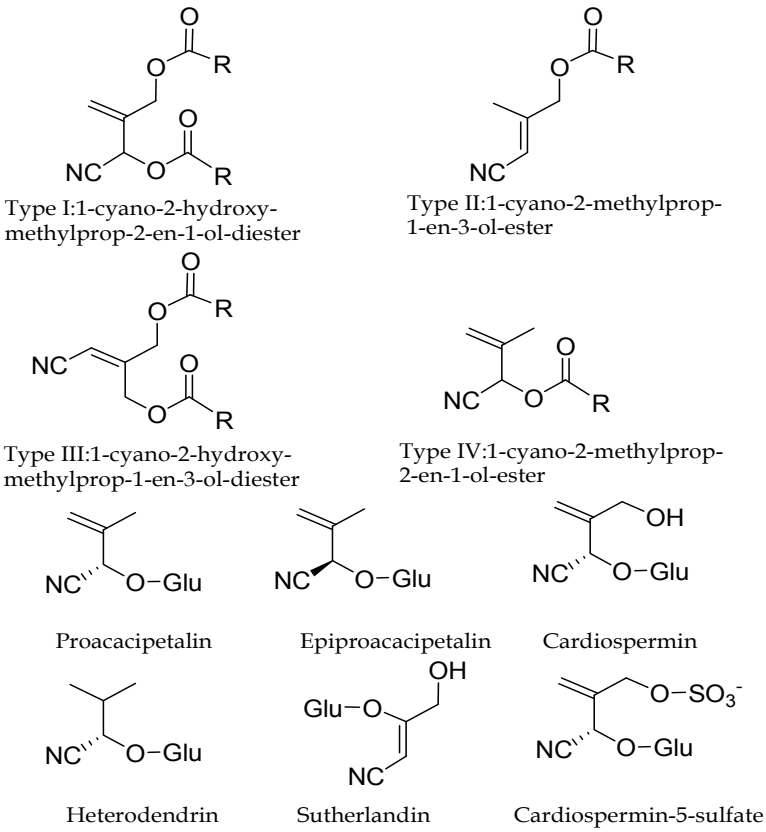


Fig. 8. Cyanolipids and glucosinolates from Sapindaceae.

Cyanolipids present in Sapindaceae belong to four types (Figure 8) which do not occur in all species. Although there is some controversy in the literature, it seems that each species has only one or two types of cyanolipids, but not all of them. The fatty acid moieties in cyanolipids vary within the species. In most of the studies, cyanolipids are esterified mostly by only one or two fatty acids. Eicosanoic acid (20:0) and eicosenoic acid (20:1 *n-9*) are present in high proportion in many species (Aichholz et al. 1997). In the genus *Paullinia*, the rare fatty acids paullinic acid (*Z*-13-eicosenoic acid) and cis-vaccenic acid (*Z*-11-octadecenoic acid) are ubiquitous constituents (Seigler 1974; Spitzer 1995; Spitzer 1996; Lago et al. 2000; Avato et al. 2003). Other fatty acids found go from dodecanoic (12:0) to docosanoic acids (22:0) (Mikolajczak et al. 1970a; 1970b; 1971; Aichholz et al. 1997; Avato et al. 2003).
Besides lipid derivatives, other cyanocompounds (the corresponding glycosides and salts) have also been isolated from aerial parts (Seigler et al. 1974; Bjarnholt et al. 2008) or roots of different species (Kumar et al. 2011). For example, cardiospermin (Figure 8) has been isolated from some species -and has been shown to be the responsible of the anxiolytic effects of ethanolic root extract from *Cardiospermum halicacabum* (Kumar et al. 2011); and in the particular case of *Cardiospermum grandiflorum* the corresponding sulphate-containing cyanogenic glucoside of cardiospermin was described (Hubel & Nahrstedt 1979).
Only cyanolipids of the type I and IV are cyanogenetic (Avato et al. 2005). Therefore cyanolipids do not work for all species as defensive compounds producing HCN. When HCN can be formed, its production works similarly to the one from glycosinolates, with a previous step of hydrolysis of the ester moieties catalyzed by estearases (Figure 9) (Wink et al. 1997). Cyanolipids can effectively work as plant defences, as it has been shown that their enzymatic breakdown produces the α-hydroxynitriles, from which HCN is released in a similar way than the one from glycosinolates (Wink et al. 1997). Moreover, it has been shown that *in vivo* HCN is produced upon wounding by herbivores (Selmar et al. 1990). Besides, as any plant defence, these defensive metabolites have been overcome in their original function by herbivores. In that sense it is well known that some Heteroptera (*Leptocorus* and *Jadera* spp.) not only are specialists on Sapindaceae, but are also able to sequester cyanolipids as such from their food plants, and biotransform them to the glycosylated derivatives (Braekman et al. 1982; Aldrich et al. 1990). Moreover, such acquisition of cyanocompounds from their host renders these gregarious, aposematic insects unpalatable to a variety of predators (Aldrich et al. 1990).

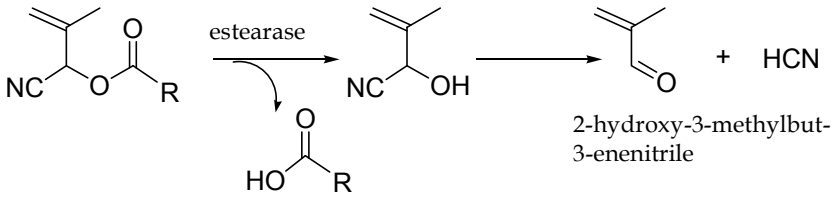


Fig. 9. Hydrolysis of cyanolipids to produce HCN (Wink et al. 1997).

Cyanolipids have been investigated in their potential as control agents for insects. In that sense, insect repellent and insecticidal properties of some of these products have been described. For instance, when adults of the red flour beetles, *Tribolium castaneum,* were exposed to seed oils from two species of *Cardiospermum* (*C. canescens* and *C. belicacabum*), the beetles preferred the arena zones where no oil was applied (Khan et al. 1983). However this repellency effect seems not to be a general pattern as in a pitfall trap bioassay, oils reach in

cyanolipids stimulated aggregation in the cases of the saw toothed grain beetle, *Oryzaephilus surinamensis*, and of the rice weevil, *Sitophilus oryzae* (Mikolajczak et al. 1984). In this later work, when tested separately the four classes of cyanolipids (Figure 8) lost their repellent activity, suggesting synergism among them for this action. When the effect of cyanolipids was tested in a contact bioassay, only the ones belonging to classes I and IV showed a paralyzing effect against the saw toothed grain beetle, *Oryzaephilus surinamensis*. Eventhough one may be tempted to correlate this effect with the capacity of producing HCN by the pure compound tested, the conclusion is not again a general one since these cyanolipids did not have any effect on three other beetles (Mikolajczak et al. 1984). Finally, the European corn borer, *Ostrinia nubilalis*, was affected in its metamorphosis when cyanolipids of the classes II and IV were incorporated in its diet (Mikolajczak et al. 1984). On the whole, even though non- glycoside cyanogens were described in Sapindaceae as early as in the 1920` (cited by Mikolajczak et al. 1969), not many studies have been carried out in regard of either their ecological role as plant defences, or their potential as biopesticides. Although in the last case one can envisioned that cyanolipids of the classes I and IV will not be selective –because HCN is generated-, there is a chance that cyanolipids of the classes II and III may have some interest in this regard.

4. Conclusion

The family Sapindaceae includes many edible species, *e.g.* ackee, rambutan, longan and lychee (fruits from *Blighia sapida, Litchi chinensis*, *Nephelium lappaceum*, *Dimocarpus longan* respectively), which are widely consumed mainly in Asia and Australia (Diczbalis 2008; Vichitrananda & Somsri 2008; Diczbalis et al. 2010). Nevertheless, some species in this family produce in different phenological stages (including fruits in some cases) bioactive compounds with medicinal or toxicological properties. With reference to insect toxicity, up to now most of the studies carried out have found activity against species in the orders Lepidoptera and Diptera (mosquitoes). However, these results may be an artefact of the biodetection itself, as much of the research focuses on chewing armyworms and borers of economically significance, and on mosquitoes as important vectors of human diseases. In addition, products from Sapindaceae have revealed differential activity on insect targets from different orders, and even from the same order (Mikolajczak et al. 1984; Khan et al. 2002; Castillo et al. 2009); and have demonstrated selectivity when their activity was checked against beneficial insects while some other products have not (Castillo et al. 2009). These findings emphasise the need for widening the spectrum of biodetectors used in the tests.

The Sapindaceae are well characterized for the presence in their seeds of toxic cyanolipids, and the occurrence of this group of secondary metabolites seems to be restricted to this family. However, it is difficult to foresee that the Sapindaceae will find their way into the development of botanicals based on their unique cyanogens due to the intrinsic general toxicity of these compounds.

This family takes its name from the soapberry tree *Sapindus saponaria* (Emanuel & Benkeblia 2011), mostly known for being rich in saponins. Those chemical constituents provide its extracts with tensoactive properties, having been widely used not only as a source of soap but also for the application of its biological effects in medicine as well as in pest control. Indeed, the tensoactivity has been a property that has found application in pest control as shown by the fact that soaps are probably among the oldest insecticides in use (Silva et al. 2007).

Many other bioactive compounds belonging to different chemical groups have also been isolated from members of this family. For instance, from species studied for their anti insect activity, it has been reported the occurrence of clerodane diterpenoids and prenylated flavonoids (*Dodonaea* spp.); flavonols, phenols and triterpenes (*Paullinia* spp.); tannins (*M. pubescens*); lectins (*Talisia esculenta)*; gallic acid, gallates and derivatives (*Acer rubrum*). Being confirmed in some cases that those compounds are the responsible for the activities found. Among these secondary metabolites, probably the ones with the strongest potential as anti-insect agents are the clerodanes. Eventhough the main sources of these diterpenes are species from Asteraceae and Lamiaceae, the Sapindaceae are showing to be also a good resource of them. So far, different studies have shown the antifeedant capability of clerodanes against many insects, including species from Lepidoptera, Coleoptera, and Orthoptera (Klein Gebbinck et al. 2002; Sosa et al. 2008).

All in all, the family Sapindaceae, which members are widely distributed in every continent and have been used since early days for different purposes –taking advantage of their medicinal and toxicological properties-, seems to be a promissory source of bioactive compounds to be used as biological control agents. However more extensive studies, not only on more species not yet prospected, but also concerning more diverse targets are still needed.

5. Acknowledgments

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6. References

Abdel, A., Shadia & Omer, E. A. (1995). Bio-evaluation of *Dodonaea viscosa* L. Jacq. extracts on the cotton leafworm, *Spodoptera littoralis* (Boisd.) as indicated by life table parameters. *Annals of Agricultural Science (Cairo)* Vol. 40, 2, pp. 891-900

Abou-Zaid, M. M., Helson, B. V., Nozzolillo, C. & Arnason, J. T. (2001). Ethyl m-digallate from red maple, *Acer rubrum* L., as the major resistance factor to forest tent caterpillar, *Malacosoma disstria* Hbn. *Journal of Chemical Ecology* Vol. 27, 12, pp. 2517-2527

Aichholz, R., Spitzer, V. & Lorbeer, E. (1997). Analysis of cyanolipids and triacylglycerols from sapindaceae seed oils with high-temperature gas chromatography and high-temperature gas chromatography chemical ionization mass spectrometry. *Journal of Chromatography A* Vol. 787, 1-2, pp. 181-194

Aldrich, J. R., Carroll, S. P., Lusby, W. R., Thompson, M. J., Kochansky, J. P. & Waters, R. M. (1990). Sapindaceae, cyanolipids, and bugs. *Journal of Chemical Ecology* Vol. 16, 1, pp. 199-210

Anonymous (2011). Germplasm Resources Information Network - (GRIN) National Germplasm Resources Laboratory, Beltsville, Maryland. In: *USDA, ARS, National*

Genetic Resources Program, 11 July 2011, Available from: http://www.ars-grin.gov/cgi-bin/npgs/html/gnlist.pl?999

Arruda, W., Cavasin Oliveira, G. M. & Garcia da Silva, I. (2003a). Morphological alterations of *Aedes aegypti* larvae exposed to *Magonia pubescens* extract. *Entomologia y Vectores* Vol. 10, 1, pp. 47-60

Arruda, W., Oliveira, G. M. C. & da Silva, I. G. (2003b). Toxicity of the ethanol extract of *Magonia pubescens* on larvae *Aedes aegypti*. *Revista da Sociedade Brasileira de Medicina Tropical* Vol. 36, 1, pp. 17-25

Arulmozhi, D. K., Veeranjaneyulu, A., Bodhankar, S. L. & Arora, S. K. (2005). Pharmacological studies of the aqueous extract of *Sapindus trifoliatus* on central nervous system: possible antimigraine mechanisms. *Journal of Ethnopharmacology* Vol. 97, 3, pp. 491-496

Avato, P., Pesante, M. A., Fanizzi, F. P. & Santos, C. A. D. (2003). Seed oil composition of *Paullinia cupana* var. *sorbilis* (Mart.) Ducke. *Lipids* Vol. 38, 7, pp. 773-780

Avato, P., Rosito, I., Papadia, P. & Fanizzi, F. P. (2005). Cyanolipid-rich seed oils from *Allophylus natalensis* and *A. dregeanus*. *Lipids* Vol. 40, 10, pp. 1051-1056

Avato, P., Rosito, I., Papadia, P. & Fanizzi, F. P. (2006). Characterization of seed oil components from *Nephelium lappaceum* L. *Natural Product Communications* Vol. 1, 9, pp. 751-755

Banerjee, H. N. (1936). Clerodin from *Clerodendron infortunatum*. *Science and Culture* Vol. 2, pp. 163

Benlekehal, H., Clotteau, M., Dornier, M. & Reynes, M. (2001). An Amazonian product particularly rich in caffeine: the seed of guarana [*Paullinia cupana* H.B.K. var. *sorbilis* (Mart.) Ducke]. *Fruits (Paris)* Vol. 56, 6, pp. 423-435

Bjarnholt, N. & Møller, B. L. (2008). Hydroxynitrile glucosides. *Phytochemistry* Vol. 69, 10, pp. 1947-1961

Boiça Junior, A. L., Medeiros, C. A. M., Torres, A. L. & Chagas Filho, N. R. (2005). Effect of plant aqueous extracts on the development of *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), on collard greens. *Arquivos do Instituto Biologico Sao Paulo* Vol. 72, 1, pp. 45-50

Bousserouel, H., Litaudon, M., Morleo, B., Martin, M. T., Thoison, O., Nosjean, O., Boutin, J. A., Renard, P. & Sevenet, T. (2005). New biologically active linear triterpenes from the bark of three Newcaledonian *Cupaniopsis* species. *Tetrahedron* Vol. 61, 4, pp. 845-851

Braekman, J. C., Daloze, D. & Pasteels, J. M. (1982). Cyanogenic and other glucosides in a neo-guinean bug *Leptocoris isolata*: Possible precursors in its host-plant. *Biochemical Systematics and Ecology* Vol. 10, 4, pp. 355-364

Bruneton, J. (1995). Pharmacognosy, phytochemistry, medicinal plants. Paris, Lavoisier Publishing: 538-544

Bullangpoti, V., Visetson, S., Milne, J. & Pornbanlualap, S. (2004). Effects of Mangosteen's peels and rambutan's seeds on toxicity, esterase and glutathione-S-transferase in rice weevil (*Sitophilus oryzae* L.). *Kasetsart Journal, Natural Sciences* Vol. 38, 5, Supplement, pp. 84-89

Castillo, L., Gonzalez-Coloma, A., Gonzalez, A., Diaz, M., Alonso-Paz, E., Bassagoda, M. J. & Rossini, C. (2009). Screening of Uruguayan plants for deterrent activity against insects. *Industrial Crops and Products* Vol. 29, 1, pp. 235-240

da Silva, I. G., Guimaraes, V. P., Lima, C. G., da Silva, H. H. G., Elias, C. N., Mady, C. M., da Mota e Silva, V. V., de Paiva Nery, A., da Rocha, K. R., Rocha, C. & Isac, E. (2003). Larvicide and toxic effect of the ethanol crude extract of the stem coat of *Magonia pubescens* towards *Aedes aegypt* in laboratory. *Revista de Patologia Tropical* Vol. 32, 1, pp. 73-86

de Sousa Araújo, T. A., Alencar, N. L., de Amorim, E. L. C. & de Albuquerque, U. P. (2008). A new approach to study medicinal plants with tannins and flavonoids contents from the local knowledge. *Journal of Ethnopharmacology* Vol. 120, 1, pp. 72-80

Deepa, B. & Remadevi, O. K. (2007). Contact toxicity of the leaf extracts of *Dodonaea viscosa* (Sapindaceae) to *Spodoptera litura* (Fab.). *Hexapoda* Vol. 14, 1, pp. 66-67

Dharmani, P., Mishra, P. K., Maurya, R., Chauhan, V. S. & Palit, G. (2005). *Allophylus serratus*: A plant with potential anti-ulcerogenic activity. *Journal of Ethnopharmacology* Vol. 99, 3, pp. 361-366

Diczbalis, Y., Nicholls, B., Groves, I. & Lake, K. (2010). Sapindaceae production and research in Australia. *Acta Horticulturae* Vol. 863, pp. 49-58

Diczbalis, Y. A. (2008). Nutrition management of tropical fruits grown in north Queensland, Australia. *Acta Horticulturae* Vol. 772, pp. 375-379

Dinesh, K. & Hasan, S. Q. Cyanolipids in sapindaceous seed oils. *Asian Journal of Chemistry* Vol. 23, 6, pp. 2589-2591

dos Santos, W. L., Freire, M. D. M., Bogorni, P. C., Vendramim, J. D. & Macedo, M. L. R. (2008). Effect of the aqueous extracts of the seeds of *Talisia esculenta* and *Sapindus saponaria* on fall armyworm. *Brazilian Archives of Biology and Technology* Vol. 51, 2, pp. 373-383

El-Din, M. M. & El-Gengaihi, S. E. (2000). Joint action of some botanical extracts against the Egyptian cotton leafworm *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae). *Egyptian Journal of Biological Pest Control* Vol. 10, 1/2, pp. 51-56

Emanuel, M. A. & Benkeblia, N. (2011). Processing of ackee fruit (*Blighia sapida* L.): present and future perspectives. *Acta Horticulturae* Vol. 894, pp. 211-213

Fernandes, F. F., D'Alessandro, W. B. & Freitas, E. P. S. (2008). Toxicity of extract of *Magonia pubescens* (Sapindales : Sapindaceae) St. Hil. to control the Brown Dog tick, *Rhipicephalus sanguineus* (Latreille) (Acari : Ixodidae). *Neotropical Entomology* Vol. 37, 2, pp. 205-208

Ferreira Barreto, C., Cavasin, G. M., Garcia da Silva, H. H. & Garcia da Silva, I. (2006). Study of the morphohistological modifications in larvae of *Aedes aegypti* (Diptera, Culicidae) submitted to the pure ethanolic extract of *Sapindus saponaria* Lin. (Sapindaceae). *Revista de Patologia Tropical* Vol. 35, 1, pp. 37-57

Gaillard, Y., Carlier, J., Berscht, M., Mazoyer, C., Bevalot, F., Guitton, J. & Fanton, L. (2011). Fatal intoxication due to ackee (*Blighia sapida*) in Suriname and French Guyana. GC-MS detection and quantification of hypoglycin-A. *Forensic Science International* Vol. 206, 1-3, pp. e103-e107

Getie, M., Gebre-Mariam, T., Rietz, R., Hohne, C., Huschka, C., Schmidtke, M., Abate, A. & Neubert, R. H. H. (2003). Evaluation of the anti-microbial and anti-inflammatory activities of the medicinal plants *Dodonaea viscosa*, *Rumex nervosus* and *Rumex abyssinicus*. *Fitoterapia* Vol. 74, 1-2, pp. 139-143

Getie, M., Gebre-Mariam, T., Rietz, R. & Neubert, R. H. H. (2002). Evaluation of the release profiles of flavonoids from topical formulations of the crude extract of the leaves of *Dodonaea viscosa* (Sapindaceae). *Pharmazie* Vol. 57, 5, pp. 320-322

Hasan, S. Q. & Roomi, Y. A. (1996). Cyanolipids in *Sapindus trifoliatus* seed oil. *Journal of the Oil Technologists' Association of India* Vol. 28, 1, pp. 23, 25

Hasan, S. Q., Roomi, Y. A. & Chitra, N. (1994). Reinvestigation of seed oil of *Sapindus saponaria* for its cyanolipid content. *Journal of the Oil Technologists' Association of India* Vol. 26, 3, pp. 77-79

Hassall, C. H. & Reyle, K. (1955). Hypoglycin-A and Hypoglycin-B, two biologically active polypeptides from *Blighia sapida*. *Biochemical Journal* Vol. 60, 1-4, pp. 334-339

Hassall, C. H., Reyle, K. & Feng, P. (1954). Hypoglycin A,B: Biologically active polypeptides from *Blighia sapida*. *Nature* Vol. 173, 4399, pp. 356-357

Hubel, W. & Nahrstedt, A. (1979). Cardiosperminsulfate - sulfur-containing cyanogenic glucoside from *Cardiospermum grandiflorum*. *Tetrahedron Letters* Vol. 20, 45, pp. 4395-4396

Isman, M., Matsuura, H., MacKinnon, S., Durst, T., Towers, G. & Arnason, J. (1996). Phytochemistry of the Meliaceae. *Recent Advances on Phytochemistry* Vol. 30, pp. 155-178

Ito, A., Chai, H. B., Kardono, L. B. S., Setowati, F. M., Afriastini, J. J., Riswan, S., Farnsworth, N. R., Cordell, G. A., Pezzuto, J. M., Swanson, S. M. & Kinghorn, A. D. (2004). Saponins from the bark of *Nephelium maingayi*. *Journal of Natural Products* Vol. 67, 2, pp. 201-205

Jain, S. C. (1976). Isolation of flavonoids from soapnut, *Sapindus emarginatus* Vahl. *Indian Journal of Pharmacy* Vol. 38, 6, pp. 141-142

Jefferie, P. R., Knox, J. R. & Scaf, B. (1973). Structure elucidation of some ent-clerodane diterpenes from *Dodonaea boroniaefolia* and *Cyanostegia angustifolia*. *Australian Journal of Chemistry* Vol. 26, 10, pp. 2199-2211

Kamala-Kannan, S., Park, S. M., Oh, B. T., Kim, H. M. & Lee, K. J. (2009). First report of aster yellows phytoplasma in Goldenrain tree (*Koelreuteria paniculata*) in Korea. *Journal of Phytopathology* Vol. 158, 3, pp. 197-199

Kanchanapoom, T., Kasai, R. & Yamasaki, K. (2001). Acetylated triterpene saponins from the Thai medicinal plant, *Sapindus emarginatus*. *Chemical & Pharmaceutical Bulletin* Vol. 49, 9, pp. 1195-1197

Kayode, J. (2006). Conservation of indigenous medicinal botanicals in Ekiti State, Nigeria. *Journal of Zhejiang University SCIENCE B* Vol. 7, 9, pp. 713-718

Khan, A. & Gumbs, F. A. (2003). Repellent effect of ackee (*Blighia sapida* Koenig) component fruit parts against stored-product insect pests. *Tropical Agriculture* Vol. 80, 1, pp. 19-27

Khan, A., Gumbs, F. A. & Persad, A. (2002). Pesticidal bioactivity of ackee (*Blighia sapida* Koenig) against three stored-product insect pests. *Tropical Agriculture* Vol. 79, 4, pp. 217-223

Khan, M. W. Y., Ahmad, F., Ahmad, I. & Osman, S. M. (1983). Nonedible Seed Oils as Insect Repellent. *Journal of the American Oil Chemists Society* Vol. 60, 5, pp. 949-950

Klein Gebbinck, E. A., Jansen, B. J. M. & de Groot, A. (2002). Insect antifeedant activity of clerodane diterpenes and related model compounds. *Phytochemistry* Vol. 61, 7, pp. 737-770

Koodalingam, A., Mullainadhan, P. & Arumugam, M. (2009). Antimosquito activity of aqueous kernel extract of soapnut *Sapindus emarginatu*s: impact on various developmental stages of three vector mosquito species and nontarget aquatic insects. *Parasitology Research* Vol. 105, 5, pp. 1425-1434

Koodalingam, A., Mullainadhan, P. & Arumugam, M. (2011). Effects of extract of soapnut Sapindus emarginatus on esterases and phosphatases of the vector mosquito, *Aedes aegypti* (Diptera: Culicidae). *Acta Tropica* Vol. 118, 1, pp. 27-36

Kumar, R., Murugananthan, G., Nandakumar, K. & Talwar, S. (2011). Isolation of anxiolytic principle from ethanolic root extract of *Cardiospermum halicacabum*. *Phytomedicine* Vol. 18, 2-3, pp. 219-223

Lago, R. C. A., Simoni, M. d. L. P. S. C. & Pinto, A. d. C. (2000). On the occurrence of cyanolipids in *Paullinia carpopodea* Cambess and *P. cupana* Kunth seed oils. *Acta Amazonica* Vol. 30, 1, pp. 101-105

Lemos, T. L. G., Mendes, A. L., Sousa, M. P. & Braz-Filho, R. (1992). New saponin from *Sapindus saponaria*. *Fitoterapia* Vol. 63, 6, pp. 515-517

Lorenzi, H. (2004). Arvores brasileiras. Manual de identificaçao e cultivo de plantas arvoreas nativas do Brasil. Piracicaba: Plantarum

Macedo, M. L. R., Damico, D. C. S., Freire, M. D., 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, 10, pp. 2980-2986

Macedo, M. L. R., Freire, M. D. M., Novello, J. C. & Marangoni, S. (2002). *Talisia esculenta* lectin and larval development of *Callosobruchus maculatus* and *Zabrotes subfasciatus* (Coleoptera : Bruchidae). *Biochimica et Biophysica Acta* Vol. 1571, 2, pp. 83-88

Mahmoud, I., Moharram, F. A., Marzouk, M. S., Soliman, H. S. M. & El-Dib, R. A. (2001). Two new flavonol glycosides from leaves of *Koelreuteria paniculata*. *Pharmazie* Vol. 56, 7, pp. 580-582

Malarvannan, S., Giridharan, R., Sekar, S., Prabavathy, V. R. & Nair, S. (2009). Ovicidal activity of crude extracts of few traditional plants against *Helicoverpa armigera* (Hubner) (Noctuidae: Lepidoptera). *Journal of Biopesticides* Vol. 2, 1, pp. 64-71

Malarvannan, S., Kumar, S. S., Prabavathy, V. R. & Sudha, N. (2008). Individual and synergistic effects of leaf powder of few medicinal plants against American bollworm, *Helicoverpa armigera* (Hubner) (Noctuidae: Lepidoptera). *Asian Journal of Experimental Sciences* Vol. 22, 1, pp. 79-88

Malarvannan, S., Sekar, S., Prabavathy, V. R. & Sudha, N. (2008). Individual and synergistic efficacy of leaf powder of *Argemone mexicana* against tobacco caterpillar, *Spodoptera litura* Fab, (Noctuidae: Lepidoptera). *Emerging trends of researches in insect pest management and environmental safety, Volume I* Vol., pp. 155-164

Malarvannan, S. & Subashini, H. D. (2007). Efficacy of *Dodonaea angustifolia* crude extracts against spotted bollworm, *Earias vitella* (Fab.) (Lepidoptera: Noctuidae). *Journal of Entomology* Vol. 4, 3, pp. 243-247

Mazzola, E. P., Parkinson, A., Kennelly, E. J., Coxon, B., Einbond, L. S. & Freedberg, D. I. (2011). Utility of coupled-HSQC experiments in the intact structural elucidation of

three complex saponins from *Blighia sapida*. *Carbohydrate Research* Vol. 346, 6, pp. 759-768

Medeiros, C. A. M., Boica Junior, A. L. & Angelini, M. R. (2007). Sub-lethal effect of aqueous vegetals extracts of *Azadirachta indica* A. Juss. and *Sapindus saponaria* L. against the biology of *Ascia monuste orseis* at kale. *Boletin de Sanidad Vegetal, Plagas* Vol. 33, 1, pp. 27-34

Meyer Albiero, A. L., Aboin Sertié, J. A. & Bacchi, E. M. (2002). Antiulcer activity of *Sapindus saponaria* L. in the rat. *Journal of Ethnopharmacology* Vol. 82, 1, pp. 41-44

Mikolajczak, K. L. (1977). Cyanolipids. *Progress in the chemistry of fats and other lipids* Vol. 15, 2, pp. 97-130

Mikolajczak, K. L., Madrigal, R. V., Smith, C. R. & Reed, D. K. (1984). Insecticidal effects of cyanolipids on three species of stored product insects, European Corn Borer (Lepidoptera: Pyralidae) Larvae, and Striped Cucumber Beetle (Coleoptera: Chrysomelidae) *Journal of Economic Entomology* Vol. 77, 5, pp. 1144-1148

Mikolajczak, K. L., Seigler, D. S., Smith Jr, C. R., Wolff, I. A. & Bates, R. B. (1969). A cyanogenetic lipid from *Cordia verbenacea* DC. Seed oil. *4* Vol. 6, 617-619,

Mikolajczak, K. L. & Smith, C. R. (1971). Cyanolipids of kusum (*Schleichera trijuga*) seed oil. *Lipids* Vol. 6, 5, pp. 349-&

Mikolajczak, K. L., Smith, C. R. & Tjarks, L. W. (1970). Cyanolipids of *Cardiospermum halicacabum* L and other sapindaceous seed oils. *Lipids* Vol. 5, 10, pp. 812-&

Mikolajczak, K. L., Smith, C. R. & Tjarks, L. W. (1970). Cyanolipids of *Koelreuteria paniculata* Laxm. seed oil. *Lipids* Vol. 5, 8, pp. 672-&

Mukherjee, S. N. & Joseph, M. (2000). Medicinal plant extracts influence insect growth and reproduction: A case study. *Journal of Medicinal and Aromatic Plant Sciences* Vol. 22-23, 4A-1A, pp. 154-158

Mukherjee, S. N. & Joseph, M. (2001). Medicinal plant extracts influence insect growth and reproduction: a case study. *Journal of Medicinal and Aromatic Plant Sciences* Vol. 22/23, 4A/1A, pp. 154-158

Murdock, L. L. & Shade, R. E. (2002). Lectins and protease inhibitors as plant defenses against insects. *Journal of Agriculture and Food Chemistry* Vol. 50, pp. 6605–6611

Muthukumran, P., Begumand, V. H. & Kalaiarasan, P. (2011). Antidiabetic activity of *Dodonaea viscosa* (L) leaf extracts. *International Journal of PharmTech Research* Vol. 3, 1, pp. 136-139

Nielsen, N. J., Nielsen, J. & Staerk, D. (2010). New resistance-correlated saponins from the insect-resistant crucifer *Barbarea vulgaris*. *Journal of Agrulture and Food Chemistry* Vol. 58, pp. 5509-5514

Niu, H. M., Zeng, D. Q., Long, C. L., Peng, Y. H., Wang, Y. H., Luo, J. F., Wang, H. S., Shi, Y. N., Tang, G. H. & Zhao, F. W. (2010). Clerodane diterpenoids and prenylated flavonoids from *Dodonaea viscosa*. *Journal of Asian Natural Products Research* Vol. 12, 1, pp. 7-14

Omosa, L. K., Midiwo, J. O., Derese, S., Yenesew, A., Peter, M. G. & Heydenreich, M. (2010). neo-Clerodane diterpenoids from the leaf exudate of *Dodonaea angustifolia*. *Phytochemistry Letters* Vol. 3, 4, pp. 217-220

Palanisamy, U., Cheng, H. M., Masilamani, T., Subramaniam, T., Ling, L. T. & Radhakrishnan, A. K. (2008). Rind of the rambutan, *Nephelium lappaceum*, a potential source of natural antioxidants. *Food Chemistry* Vol. 109, 1, pp. 54-63

Pelegrini, D. D., Tsuzuki, J. K., Amado, C. A. B., Cortez, D. A. G. & Ferreira, I. C. P. (2008). Biological activity and isolated compounds in *Sapindus saponaria* L. and other plants of the genus *Sapindus*. *Latin American Journal of Pharmacy* Vol. 27, 6, pp. 922-927

Pérez, D. & Iannacone, J. (2004). Insecticidal effect of *Paullinia clavigera* var. bullata simpson (Sapindaceae) and *Tradescantia zebrina* Hort. ex Bosse (Commelinaceae) in the control of *Anopheles benarrochi* Gabaldon, main vector of malaria in ucayali, Peru *Ecología Aplicada* Vol. 3, pp. 64-72

Pérez, D., Iannacone, J. & Pinedo, H. (2010). Toxicological effect from the stem cortex of the amazonic plant soapberry *Paullinia clavigera* (Sapindaceae) upon three arthropods. *Ciencia E Investigacion Agraria* Vol. 37, 3, pp. 133-143

Pérez, D., Iannacone, J. & Tueros, A. (2008). Toxicity of *Paullinia clavigera* schltdl. (Sapindaceae) and *Chondrodendron tomentosum* ruiz et pav. (Menispermaceae) on jumping lice of camu camu *Tuthillia cognata* (Hemiptera: Psyllidae). *Gayana Botanica* Vol. 65, 2, pp. 145-152

Pérez, D. D. & Iannacone, O. J. (2006). Effectiveness of botanical extracts from ten plants on mortality and larval repellency of *Rhynchophorus palmarum* L., an insect pest of the Peach palm *Bactris gasipaes* Kunth in Amazonian Peru. *Agricultura Tecnica* Vol. 66, 1, pp. 21-30

Peumans, W. J. & Van Damme, E. J. M. (1995). Lectins as plant defense proteins. *Plant Physiology* Vol. 109, pp. 347-352

Plasman, V., Plehiers, M., Braekman, J., Daloze, D., De Biseau, J. & Pasteels, J. (2001). Chemical defense in *Platyphora kollari* Baly and *Leptinotarsa behrensi* Harold (Coleoptera: Chrysomelidae). Hypotheses on the origin and evolution of leaf beetles toxins. *Chemoecology* Vol. 11, pp. 107-112

Porras, M. F. & Lopez-Avila, A. (2009). Effect of extracts from *Sapindus saponaria* on the glasshouse whitefly *Trialeurodes vaporariorum* (Hemiptera: Aleyrodidae). *Revista Colombiana De Entomologia* Vol. 35, 1, pp. 7-11

Prieto, J. M., Schaffner, U., Barker, A., Braca, A., Siciliano, T. & Boevé, J. L. (2007). Sequestration of furostanol saponins by *Monophadnus* sawfly larvae. *Journal of Chemical Ecology* Vol. 33, 513-524,

Rahman, S. S., Rahman, M. M., Begum, S. A., Khan, M. M. R. & Bhuiyan, M. M. H. (2007). Investigation of *Sapindus mukorossi* extracts for repellency, insecticidal activity and plant growth regulatory effect. *Journal of Applied Sciences Research* Vol., February, pp. 95-101

Rey, D., Pautou, M.-P. & Meyran, J.-C. (1999). Histopathological effects of tannic acid on the midgut epithelium of some aquatic Diptera larvae. *Journal of Invertebrate Pathology* Vol. 73, 2, pp. 173-181

Ribeiro, A., Zani, C. L., Alves, T. M. D., Mendes, N. M., Hamburger, M. & Hostettmann, K. (1995). Molluscicidal saponins from the pericarp of *Sapindus saponaria*. *International Journal of Pharmacognosy* Vol. 33, 3, pp. 177-180

Rodrigues, A. M. S., De Paula, J. E., Degallier, N., Molez, J. F. & Espindola, L. S. (2006). Larvicidal activity of some Cerrado plant extracts against *Aedes aegypti*. *Journal of the American Mosquito Control Association* Vol. 22, 2, pp. 314-317

Rodriguez, E. M. (1958). The wood of Argentine Sapindaceae. Structure, characteristics, and applications. *Rev Fac Agron Y Vet [Buenos Aires]* Vol. 14, (2), pp. 271-305

Saha, S., Walia, S., Kumar, J., Dhingra, S. & Parmar, B. S. (2009). Screening for feeding deterrent and insect growth regulatory activity of triterpenic saponins from *Diploknema butyracea* and *Sapindus mukorossi*. *Journal of Agricultural and Food Chemistry* Vol. 58, 1, pp. 434-440

Sarita, R., Geeta, N., Srivastava, V. K., Hasan, S. N. & Hasan, S. Q. (2002). Cyanolipids in *Sapindus obovatus* seed oil and reinvestigation of the seed oil of *Heliotropium indicum*, *H. eichwaldi* and *D. viscosa*. *Journal of the Oil Technologists' Association of India* Vol. 34, 2, pp. 69-71

Seigler, D. (1974). Determination of cyanolipids in seed oils of the Sapindaceae by means of their NMR spectra. *Phytochemistry* Vol. 13, 5, pp. 841-843

Seigler, D. S. (1976). Cyanolipids in *Cordia verbenacea*: A correction. *Biochemical Systematics and Ecology* Vol. 4, 4, pp. 235-236

Seigler, D. S. (1991). Cyanide and cyanogentic glycosides. In: *Herbivores: Their Interactions with Secondary Plant Metabolites*, Rosenthal, G. A. & Berembaum, M. R., pp. Academic Press San Diego, California

Seigler, D. S., Eggerding, C. & Butterfield, C. (1974). A new cyanogenic glycoside from *Cardiospermum hirsutum*. *Phytochemistry* Vol. 13, 10, pp. 2330-2332

Seigler, D. S., Mikolajczak, K. L., Smith Jr, C. R. & Wolff, I. A. (1970). Structure and reactions of a cyanogenetic lipid from *Cordia verbenacea* DC. seed oil. *Chemistry and Physics of Lipids* Vol. 4, pp. 147-161

Selmar, D., Grocholewski, S. & Seigler, D. S. (1990). Cyanogenic lipids: Utilization during seedling development of *Ungnadia speciosa*. *Plant Physiology* Vol. 93, 2, pp. 631-636

Sharaby, A., Abdel-Rahman, H. & Moawad, S. (2009). Biological effects of some natural and chemical compounds on the potato tuber moth, *Phthorimaea operculella* Zell. (Lepidoptera: Gelechiidae). *Saudi Journal of Biological Sciences* Vol. 16, 1, pp. 1-9

Sharma, A., Sati, S. C., Sati, O. P., Sati, D. M. & Kothiyal, S. K. (2011). Chemical constituents and Bioactivities of genus *Sapindus*. *International Journal of Research in Ayurveda & Pharmacy* Vol. 2, 2, pp. 403-409

Silva, H. H. G. d., Silva, I. G. d., dos Santos, R. M. G., Rodrigues Filho, E. & Elias, C. N. (2004). Larvicidal activity of tannins isolated of *Magonia pubescens* St. Hil. (Sapindaceae) against *Aedes aegypti* (Diptera, Culicidae). *Revista da Sociedade Brasileira de Medicina Tropical* Vol. 37, 5, pp. 396-9

Silva, T. M. S., Da Silva, T. G., Martins, R. M., Maia, G. L. A., Cabral, A. G. S., Camara, C. A., Agra, M. F. & Barbosa, J. M. (2007). Molluscicidal activities of six species of Bignoniaceae from north-eastern Brazil, as measured against *Biomphalaria glabrata* under laboratory conditions. *Annals of Tropical Medicine and Parasitology* Vol. 101, 4, pp. 359-365

Simpson, B., Claudie, D., Smith, N., Wang, J. P., McKinnon, R. & Semple, S. (2010). Evaluation of the anti-inflammatory properties of *Dodonaea polyandra*, a Kaanju traditional medicine. *Journal of Ethnopharmacology* Vol. 132, 1, pp. 340-343

Sofidiya, M. O., Jimoh, F. O., Aliero, A. A., Afolayan, A. J., Odukoya, O. A. & Familoni, O. B. (2008). Antioxidant and antibacterial properties of *Lecaniodiscus cupanioides*. *Research Journal of Microbiology* Vol. 3, 2, pp. 91-98

Sosa, M. E. & Tonn, C. E. (2008). Plant secondary metabolites from Argentinean semiarid lands: bioactivity against insects. *Phytchemical reviews* Vol. 7, 1, pp. 3-24

Sousa, S. A., Alves, S. F., de Paula, J. A. M., Fiuza, T. S., Paula, J. R. & Bara, M. T. F. (2009). Determination of tannins and methylxanthines in powdered guarana (*Paullinia cupana* Kunth, Sapindaceae) by high performance liquid chromatography. *Brazilian Journal of Pharmacognosy* Vol. 20, 6, pp. 866-870

Spitzer, V. (1995). GLC-MS analysis of the fatty-acids of the seed oil, triglycerides, and cyanolipid of *Paullinia elegans* (Sapindaceae): A rich source of cis-13-eicosenoic acid (paullinic acid). *Journal of High Resolution Chromatography* Vol. 18, 7, pp. 413-416

Spitzer, V. (1996). Fatty acid composition of some seed oils of the Sapindaceae. *Phytochemistry* Vol. 42, 5, pp. 1357-1360

Teffo, L. S., Aderogba, M. A. & Eloff, J. N. (2010). Antibacterial and antioxidant activities of four kaempferol methyl ethers isolated from *Dodonaea viscosa* Jacq. var. *angustifolia* leaf extracts. *South African Journal of Botany* Vol. 76, 1, pp. 25-29

Teixeira, J. R. M., Lapa, A. J., Souccar, C. & Valle, J. R. (1984). Timbós: ichthyotoxic plants used by brazilian indians. *Journal of Ethnopharmacology* Vol. 10, 3, pp. 311-318

Ucciani, E., Mallet, J. F. & Zahra, J. P. (1994). Cyanolipids and fattyacids of *Sapindus trifoliatus* (Sapindaceae) seed oil. *Fat Science Technology* Vol. 96, 2, pp. 69-71

Van Damme, E. J. M., Lannoo, N., Peumans, W. J. & Jean-Claude Kader and Michel, D. (2008). Plant Lectins. In: *Advances in Botanical Research*, pp. 107-209 Academic Press

Veeramani, C., Pushpavalli, G. & Pugalendi, K. V. (2010). In vivo antioxidant and hypolipidemic effect of *Cardiospermum halicacabum* leaf extract in streptozotocin-induced diabetic rats. *Journal of Basic and Clinical Physiology and Pharmacology* Vol. 21, 2, pp. 107-125

Vichitrananda, S. & Somsri, S. (2008). Tropical fruit production in Thailand. *Acta Horticulturae* Vol. 787, pp. 33-46

Waako, P. J., Gumede, B., Smith, P. & Folb, P. I. (2005). The in vitro and in vivo antimalarial activity of *Cardiospermum halicacabum* L. and *Momordica foetida* Schumch. et Thonn. *Journal of Ethnopharmacology* Vol. 99, 1, pp. 137-143

Weckerle, C. S., Stutz, M. A. & Baumann, T. W. (2003). Purine alkaloids in *Paullinia*. *Phytochemistry* Vol. 64, 3, pp. 735-742

Wilbert, W. & Haiek, G. (1991). Phytochemical screening of a Warao pharmacopoeia employed to treat gastrointestinal disorders. *Journal of Ethnopharmacology* Vol. 34, 1, pp. 7-11

Wink, M., Dey, P. M. & Harborne, J. B. (1997). Special nitrogen metabolism. In: *Plant Biochemistry*, pp. 439-486 Academic Press London

Wollenweber, E. & Roitman, J. N. (2007). New reports on surface flavonoids from *Chamaebatiaria* (Rosaceae), *Dodonaea* (Sapindaceae), *Elsholtzia* (Lamiaceae), and *Silphium* (Asteraceae). *Natural Product Communications* Vol. 2, 4, pp. 385-389

Zhao, H., Qin, X. & Yang, M. (2006). Preliminary study contact toxicity of extracts from *Dodonaea viscosa* seed aganist *Sitophilus oryzae*. *Journal of Yunnan Agricultural University* Vol. 22, 6, pp. 942-944

Zidan, Z. H., Gomaa, A. A., Afifi, F. A., Fam, E. Z. & Ahmed, S. M. S. (1993). Bioresidual activity of certain plant extracts on some stored grain insects, in relation to seed viability. *Arab Universities Journal of Agricultural Sciences* Vol. 1, 1, pp. 113-123

14

Pest Management Strategies for Potato Insect Pests in the Pacific Northwest of the United States

Silvia I. Rondon
Oregon State University, Hermiston Agricultural Research and Extension Center, Hermiston, OR, USA

1. Introduction

This publication addresses pest management guidelines for insects that attack potatoes in the Pacific Northwest of the United States (Fig. 1). Non-chemical control options are strongly encouraged; however, proper use of insecticides has proven effective when used as an additional tool in integrated pest management programs. In general terms, Integrated Pest Management (IPM) is defined as a comprehensive approach of pest control (the term pest includes insects, weeds, diseases) that when combined, reduces the number of pest densities to a level tolerable by the crop. Traditional management practices include the use of host-plant resistance, cultural, mechanical, biological and chemical means of control. Overall, scientific research-based knowledge is required to implement a successful and functional management program.

Fig. 1. The Pacific Northwest includes Washington and Oregon in the U.S. and the Canadian province of British Columbia. Geographical limits are indicated: California (south), the Pacific Ocean (west), and Idaho (east).

Insects, both as pests and beneficials, have been studied by entomologists for a very long time. Of the millions of insects on the planet, only a fraction causes problems for crops and humans. Specific to this discussion, fewer than a dozen species cause most of the insect damage to potato production in the Pacific Northwest. Many of these pests infest at a specific stage of crop growth (e.g., seed corn maggot damage is more severe at planting), while others are permanent residents before planting, at planting and during crop development (e.g., wireworms) (Fig. 2). A number of pests are important to all western U.S. regions such as aphids, especially green peach aphid and potato aphid, both important vectors of leafroll virus, and more recently PVY. Other pests are important in specific regions such as beet leafhoppers in northeastern Oregon and southeastern Washington. This chapter will briefly provide a general profile of the potato crop in the region and will also discuss the most important insect pests affecting potato production. A general description of the pest, biology, ecology, monitoring and control will be provided. No specific recommendations are made regarding insecticide compounds. To obtain more information refer to the most current version of the Pacific Northwest Insect Control Handbook located at http://uspest.org/pnw/insects or consult your local extension office.

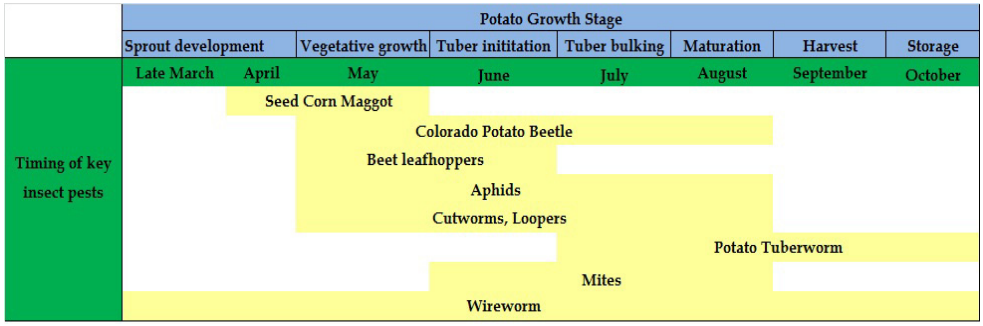

Fig. 2. Occurrence of potato insect pests in the Pacific Northwest of the United States.

2. Profile of the potato crop

The potato is one of the world's most important food crops, domesticated approximately 8,000-10,000 years ago by native Peruvians in South America (Hawkes, 1990; Ministry of Agriculture, 2008). The potato arrived in Europe in the late 1500s (Salaman, 1985) and since then potatoes have been widely cultivated in Europe, Africa, Asia/Oceania and North America. The top ten potato producers worldwide are China, Russia, India, United States, Ukraine, Poland, Germany, Belarus, Netherlands, and France (http://www.potato2008.org/en/world/index.html; source http://faostat.fao.org/default .aspx).

In the U.S., all 50 states cultivate potatoes; however, including Idaho, the Pacific Northwest produces nearly two thirds of the potatoes grown in North America. Idaho is consistently ranked first in potato production and acreage (28% of total U.S. production), followed by Washington State (23% of total U.S. production). Other top U.S. potato producing areas are Oregon, California, Montana, Colorado, North Dakota, Michigan, Wisconsin, and Maine (Fig. 3) (http://www.agcensus.usda.gov/index.asp). Florida and Texas can be included as leading farm cash receipts (http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1235).

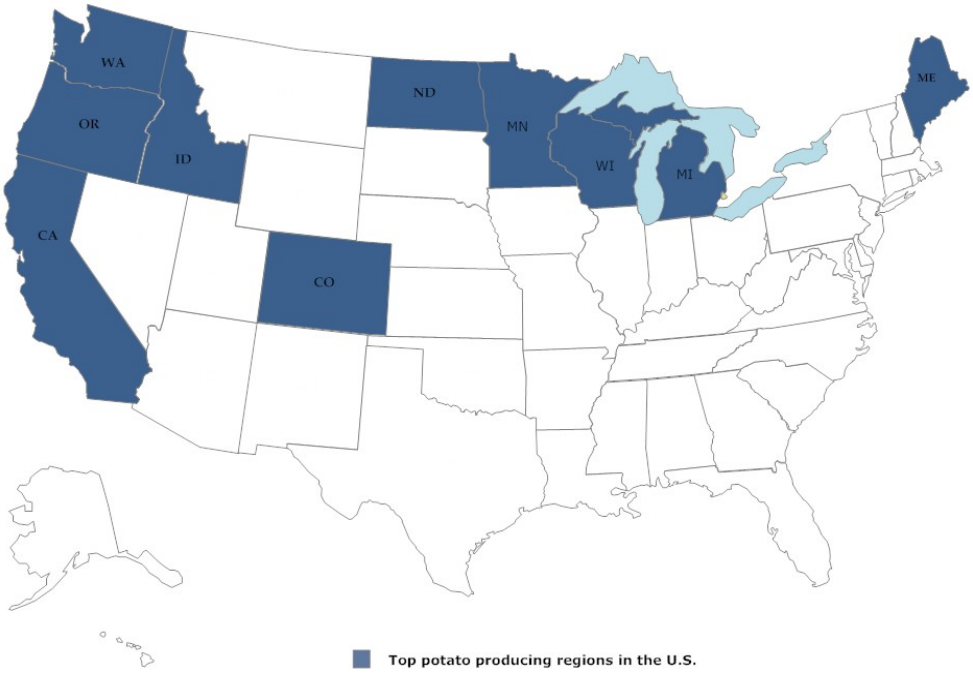

Fig. 3. Top potato producing regions in the U.S.

In the Pacific Northwest, potatoes are typically planted in the spring and harvested in late summer and fall. The growing season in most areas ranges from 120 to 140 days although high elevation areas have a 90 to 100 day season. In the Columbia Basin of Washington and Oregon, one of the most prolific regions in North America, potato production ranges between 160 to 180 days (University of California, 1986). Potatoes are produced as annual plants that grow from specialized underground stems called stolons later known as tubers (Gopal & Khurana, 2006). This vegetative propagation through the planting of tubers or part of tubers (seed pieces) is a common practice in the region (University of California, 1986; Wale et al., 2008). "Russet Burbank" is still a common variety in the region, followed by other Russet varieties such as Ranger Russet and Russet Norkotah (Table 1). Other varieties are available to producers in the region; many originated from the Tri-State potato breeding program (http://potatoes.wsu.edu/varieties/key.html). The Tri-State Program was created in the mid 1980's to intertwine researchers, scientists and stakeholders in the Pacific Northwest to promote and develop new potato varieties.

Thirty-seven Tri State releases has been released since the mid 1980's including the popular "Premier Russet", Western Russet" (both dual purposes fry and fresh packs), "Blazer Russet", "Umatilla Russet", and "Ranger Russet" (processing for fries), and "Alturas" (dehydration/processing) (Potato Variety Management Institute http://www.pvmi.org/varieties/varieties.htm).

Varieties	Year										
	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007
Russet Burbank	80	78	74	75	71	71	69	63	63	66	62
Russet Norkotah	5	5	8	8	8	8	10	14	12	10	10
Ranger Russet	7	6	4	4	4	3	1	2	1	-	1
Shepody	4	7	9	8	11	12	13	13	15	13	14
Umatilla	-	-	-	1	-	-	-	-	-	-	2
Alturas	-	-	-	-	-	-	1	3	3	2	2
Others	4	5	4	4	6	6	5	5	6	9	9

Table 1. Popular potato varieties (%) planted in Idaho, Washington and Oregon (Washington Potato Commission, 2007; National Agricultural Statistics Service, http://www.agcensus.usda.gov/index.asp).

After producers determine the nutritional needs of the crop (nitrogen, phosphorous, potassium), seed potatoes are cut uniformly, treated with fungicides and/or insecticides, and planted. Potatoes emerge 3-5 weeks after planting, depending on weather conditions, location and time of the year. After plant emergence, producers monitor crop needs regarding fertilization, pest control, and general management. Most potatoes in the area are grown under irrigation. Another common practice is desiccation or vine-killing (Hutchinson & Stall, 2007). Timely vine killing is essential for good tuber skin set, and efficient harvest. It is not clear if desiccation (naturally or artificially) of early season potatoes influences pest pressure in other nearby fields awaiting harvest. Johnson (2008) suggests a holistic plan for potato management combining management practices in the years prior to growing the potato crop, during the growing season, at harvest and during tuber storage. Miller and Hopkins (2008) specified that among many things, establishing appropriate long-term crop rotation, applying and alternating pesticides with different mode of action to avoid pesticide resistance, and improving soil microbial, physical and chemical health are necessary to achieve a healthy crop.

The potato plant develops through four clearly defined growth stages: (1) vegetative growth, (2) tuber initiation, (3) tuber growth and (4) plant maturation (Johnson, 2008). Each stage is affected by different groups of insect pests. The degree of the damage will depend on the timing of events, cultivar characteristics, and the intrinsic characteristics of each pest.

3. Managing insect pests in potatoes

The first step for managing insect pests is the timely detection of pest infestation. The goal is to manage the crop as a whole system, keeping pests at acceptable levels by utilizing several harmonizing strategies (Johnson, 2008). Earlier in this chapter, IPM was defined as a comprehensive pest control. Heitefuss (1989) defines IPM as a system in which all economically, ecologically and toxicologically suitable procedures are utilized in maximum harmony to maintain pests below the economic threshold. In the late 1990's, Luckmann and Metcalf (1994) defined IPM as an intelligent selection and use of pest control actions (or tactics) that will ensure favorable economic, ecological, and sociological consequences. IPM techniques include monitoring of pest populations, the judicious use of pesticides, and the

effective communication regarding the necessity of implementing a control tactic or not. In a modern definition, the goal is to prevent and suppress pests with minimum impact on human health, the environment and non-target organisms (Dreistadt, 2004). According to Pedigo (1986), in order to use an IPM approach, an understanding of the following factors is required: (1) the biology of the pest and its natural enemies, (2) the response of crops to management practices, (3) the effect of pesticide application on pests and non-target organisms, and (4) the action threshold or level of damage tolerable by the plant.

3.1 Monitoring

Timing detection and monitoring is an essential long-term requirement against pest infestation in potatoes. Plants should be regularly checked for signs and symptoms of pest damage. During the height of the growing season, plants should be checked once or twice per week. The inspection of the undersides of leaves and the inner plant canopy is recommended since many pests prefer sheltered sites. A close look of any plant that has missing, absent or damaged leaves or flowers, or plants whose color, texture, or size looks different than healthy ones, can be a signs of a problem.

Some insect pests can be dislodged by laying a sheet, sturdy cardboard or paper below the infested plants and "beating" the canopy. Insects can be monitored with a hand lens or traps. Both techniques not only allow detection and monitoring of pest problems but also provide estimates of pest population density. The optimal timing of sampling depends upon the life history and behavior patterns of the pest and/or beneficial insects, as well as crop stage and state, and also environmental conditions. Some areas, such as field edges or fields next to main roads are more prone to pest problems (personal observations).

Different sampling procedures can be used depending on the crop, size of field, etc. In the Pacific Northwest, the majority of potatoes are planted in circles, under center-pivot irrigation, thus fields can be divided in quarters (e.g, north, east, west, south). Upon entering the field, quick visual examination of the field is recommended. Uncharacteristic areas with poor stands or patchy growth should be scouted thoroughly. In the Pacific North-western region traps are widely used. Some traps can be baited with a pheromone which is a chemical that usually attracts a single species (e.g., potato tuber moths). Traps can also be coated with adhesive material to "stick" the pest to the trap. Either natural (e.g., virgin females) or more often the synthetic pheromones are used to attract males (Roelofs et al., 1975; Persoons et al,. 1976; Voerman & Rothschild, 1978; Rothschild, 1986; Raman, 1988). Pheromones specifically disrupt the reproductive cycle of harmful insects. Pheromone traps are used extensively in commercial agriculture in the region helping farmers detect the presence of pest species (Merrill et al., 2011). Traps in general will work only for adult insects, as adults have developed wings and are more mobile. Yellow sticky traps attract fruit flies, winged aphids, thrips, psyllids, fungus gnats, wasps, numerous flies; many species collected in sticky cards belong to the same family or group (e.g., several moths in the Gelechiidae family can be found in traps meant to be for tuber moths, a member of the Gelechiidae family), thus correct identification of target pest(s) is needed.

3.2 Management

Before planting, selecting field location can minimize pest damage and improve predictability of pest problems (Hoy et al., 2008). Also circumventing planting dates when insects will emerge and inflict most damage is recommended. Certified seed should be used at all times. If available, the selection of cultivars resistant or tolerant to important pests is also desirable.

3.2.1 Seed quality and certification

High quality seed is essential for the production of a profitable crop (Love et al., 2003). Several diseases, some transmitted by insects and especially aphids, can be transmitted to infected seed such as viruses, bacterial ring rot, blackleg, late blight, scab, and wilt diseases (University of California, 1986). Disease-free seed tubers are available from certified nurseries where seed potatoes are grown for several generations before being commercially offered to producers. This process is regulated by each state's seed certification programs. The use of appropriate production practices is only half of the process of growing high quality potatoes (Love t al. 2003). Samples from seed growing areas are grown in field trial to establish disease problems. There is a range of tolerance from zero to 6% for certain viruses (University of California, 1986). For instance, Idaho inspection tolerance for post-harvest winter tests of seed destined for recertification range from 0.8% for leafroll, 2.0% mosaic, and 5% chemical injury (Love et al., 2003). Thus, (1) clean all equipment thoroughly before entering fields, (2) establish aphid control programs to prevent the development of aphid populations in potato seed fields, (3) control aphids in potato fields to prevent the movement of aphids to seed fields.

3.2.2 Resistant cultivars

Cultivars tolerant or resistant to pests can provide long-term protection for the potato crop. For a long time it has been recognized that the evaluation of potato germplasm for resistance is a valuable tool to developing IPM programs (Horgan et al., 2007). Although no commercial potato cultivar is completely resistant to insect damage, there are variations in the susceptibility of cultivars to the pest (Hoy et al., 2008). For example, tubers of the transgenic clone Spunta G2 are resistant to the potato tuberworm (Douches et al., 2002). Rondon et al., (2009) tested several cultivars including Spunta G2 with excellent results. However the public perception regarding genetically-modified organisms is still "blocking" the widely use of this type of resources.

3.2.3 Cultural practices

Proper management of field preparation, planting, harvesting and storage are essential for maximum yield and tuber quality (University of California, 1986). Pest infestation can be minimized by avoiding contaminated seed tubers, soil, and water, sanitation of machinery brought from infested areas, removing cull piles, and following rational fertilizer and irrigation programs. Crop rotation, which reduces certain pests by breaking their life cycle, should be an integral part of a holistic management program.

3.2.4 Biological control

In a sustainable ecosystem, insect pest populations may be kept in check by natural enemies such as other insects. Parasitoids, predators, pathogens, antagonists, or competitor populations that suppress pest populations are desirable in fields (Driesche & Bellows, 1996). Under current pest management programs in potatoes, especially with an intensive agricultural production system centered on frequent calendar sprays of broad-spectrum insecticides, the impact of natural enemies is relatively unknown (Koss, 2003; Rondon, 2010). In contrast, a lot of information regarding the biology and the potential of natural enemies (a.k.a., biological control agents) can be found in the literature (Rondon, 2010). The advantage of using biological control agents is that they have no pre-harvest intervals, and are safer for application personnel, consumers and non-target organisms.

In the Pacific Northwest region, a number of arthropod species attack insect pests at the egg, immature, and/or adult stage. Species of Heteroptera (former Hemiptera, Family Pentatomidae) such as *Podisus maculiventris* (Say) (spined soldier bug) and the *Perillus bioculatus* (Fabricius) (two-spotted stink bug), *Opolomus dichrosus* L. (no common name) can be found in potato fields feeding on *Leptinotarsa decemlineata* (Say) (Colorado potato beetle) (Ferro, 1994; Lacey et al., 2001). Tamaki et al., (1983) and Lopez et al., (1995) indicated that *Myiopharus doryphorae* L. (Tachinidae fly) and *Edovum puttleri* L. (Eulophidae wasp) are moderately efficient parasitoids of Colorado potato beetles. Nabidae (damsel bugs), Neuroptera (lace wings), Coccinellidae (lady bugs), Carabidae (ground beetles) and spiders are also common inhabitants in potato fields behaving as generalists feeding on aphids, thrips, small larvae and potato beetles.

3.2.5 Chemical methods

Traditional IPM text books recommend considering chemical controls only if other techniques do not result in adequate pest control. However, chemical controls can be effectively used with other techniques (e.g., cultural, physical, biological, etc.). When choosing a chemical, be sure to read the label and choose the right product; chemical selection can be the breaking point for success or failure in controlling target pests. Worldwide, the potato requires more pesticides than any other major food crop (CIP, 1994). Intense pesticide dependency has led to the development of pesticide resistance in cases such as the Colorado potato beetles in almost all potato-growing areas in the U.S. (Weisz et al.,1995). To date, the only exception of documented Colorado potato resistance to chemicals is in the Pacific Northwest (Schreiber et al., 2010). Management tactics that preserve susceptible genotypes and allow them to interbreed with resistant individuals have the greatest potential for resistance management according to Tabashnik (1989) and Weisz et al., (1996).

4. Potato pests in the Pacific Northwest

4.1 Wireworms (Order Coleoptera: Family Elateridae)

Wireworms are one of the most destructive insect pests in the Pacific Northwest. Nearly 40 species from 12 genera attack potato, but only a few are economically important (Hoy et al., 2008). In irrigated production, the most common wireworm species are the Pacific Coast wireworm *(Limonius canus* LeConte*)*, the sugar beet wireworm *(L. californicus* (Mannerheim)), the western field wireworm (*L. infuscatus* Motschulsky*)*, and the Columbia Basin wireworm (*L. subauratus* LeConte*)*. Areas with annual rainfall less than 15 inches may be infested with the Great Basin wireworm (*Ctenicera pruinina* (Horn)). West of the Cascade mountains *Agriotes* spp. is the most common pest. A complex of species may occur (Figure 4) (Andrew et al., 2008).

4.1.1 Pest description

Wireworms are the larval stage of click beetles. Adult click beetles are slender hard-shelled insects. They range in color from chocolate to dark brown and from about 0.8 – 1.9 cm long, depending on species. Click beetles get their name from their ability to snap a spine on their thorax that produces a "clicking" sound and allows them to jump in the air when distressed or disturbed. All beetles in the Elateridae family have this ability. This technique is used to avoid predation or to get back on their feet after falling on their backs. Depending on the

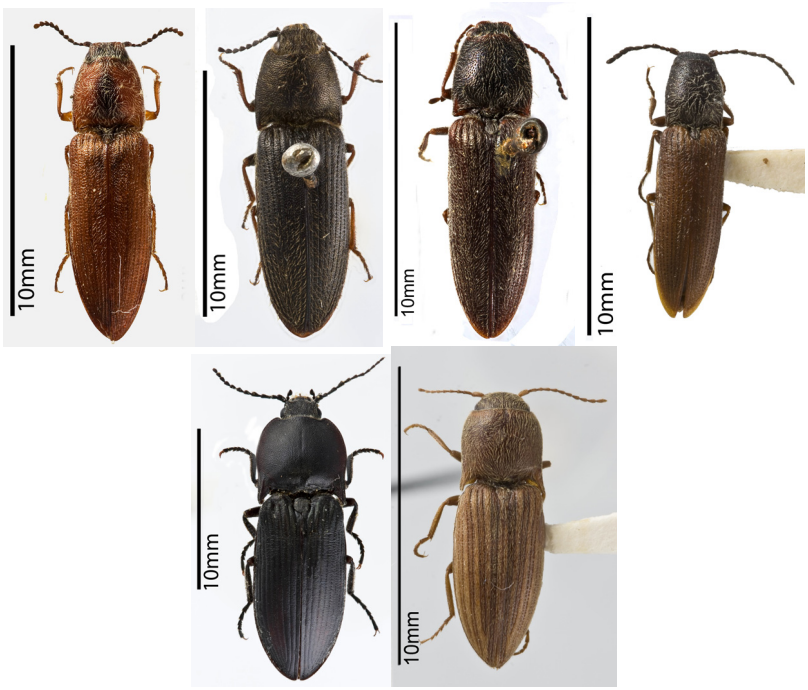

Fig. 4. Wireworms in the Pacific Northwest, from left: *Limonius canus*, *L. californicus*, *L. infuscatus*, *L. subauratus*, *Ctenicera pruinina*, and *Agriotes lineatus* (Photo by C.J. Marshall, Oregon State Arthropod Collection http://osac.science.oregonstate.edu).

species, each female after mating lays an average of 80 eggs, singly or in small clusters in the soil. Immature stages have a hardened and shiny shell and very few hairs (Fig. 5). They have three body regions with a distinct head, a thorax with 3 pairs of legs and a segmented abdomen with a tail-end (Jensen et al., 2011). Characteristics of the tail-end serve for identification purposes. Depending on species and age, wireworm larvae range from about 2 mm after hatching to 4 cm long or more at maturity. Wireworm pupae are first white, but later change to reddish-brown; they pupae in the soil.

Fig. 5. Wireworm larva (Photo by A. Jensen, Washington Potato Commission).

4.1.2 Damage

Wireworms can cause damage to potatoes by feeding upon potato seed pieces and sprouts in the spring, facilitating infection by pathogens or other insect pests. The latter damage can result in reduction in yield and/or rejection of the entire crop. In the U.S. there is zero tolerance for live larvae in tubers. Wireworms tend to be most damaging in potatoes that follow corn or small grains (wheat, barley) and on ground just entering cultivation. Wireworms damage potatoes both near planting time (damage to seed pieces) and during the growing season (damage to developing tubers). They can also be a problem at harvest and before entering storage.

4.1.3 Hosts

Potatoes, corn, wheat and grass are hosts for several species of wireworms in the Pacific Northwest. Also, beans, carrots, peas, and other annual crops may be infested; while melons, beet roots, and strawberry fruits are affected less frequently.

4.1.4 Biology

Adult click beetles emerge from pupae in the soil from late spring through late summer. In the Pacific Northwest, wireworms overwinter as larvae or adults. They can have up to a 7-year life cycle from egg to adult. Adults can fly, but usually remain in the areas where they developed as larvae. Eggs are laid in grassland or in cereal crops where larvae feed on grass roots and overwinter in the soil. Females tend to lay eggs in grassy areas. Larvae can live from 2-5 years in the soil, depending on the species. They require several years to mature and can overwinter at a depth of 30-100 cm or more in the soil, only to return near the surface in spring to resume feeding when soil temperatures exceed 50°F (10°C). Later in the season when temperatures reach 80°F (26.6°C) and above, the larvae tend to move deeper than 15 cm into the soil to escape the "heat" (Hoy et al., 2008; Schreiber et al., 2010). Wireworm larva can be confused with larvae of the family Tenebrionidae (false wireworms) or Tipulidae (crane fly). The Tenebrionidae group tends to be saprophagous while Tipulidae are associated with grassy crops.

4.1.5 Monitoring

Trapping should start early, especially in areas with history of wireworm problems. In the Pacific Northwest trapping starts mid to late March until April to May. Horton (2006) modeled the relationship between bait trap counts and crop damage by *L. canus* in Wapato, WA. Horton's model predicts tuber damage based on number of wireworms collected. Wireworm presence or absence in a field should be determined before using control measures. Unfortunately, current monitoring methods are time consuming, laborious and often do not accurately reflect field populations of this pest. Historically, wireworms have been monitored by extracting and sifting through soil cores to locate larvae. Since the distribution of wireworms in a field tends to be patchy and unpredictable, large numbers of samples are required to accurately estimate population size. Baits have largely replaced random soil sampling, since they are less labor intensive and may detect low wireworm populations. Baited traps can be constructed by placing 3-4 tablespoons of a mixed of wheat and corn seeds or rolled oats inside a fine mesh bag or nylon. The seed mixture should be soaked in water 24 hours prior to placement in the hole to facilitate germination. Dig a hole about 20-25 cm deep and 3.5- 4 cm wide at the soil surface (Horton, 2006). Bury the mixture

at the bottom of the hole. Fill the hole and mound a "soil dome" over the covered bait to serve as a solar collector and to prevent standing water. Cover each mound with a sheet of black plastic and cover the edges with soil to hold the plastic sheet down. The plastic collects solar radiation and speeds germination of the mixture. The germinating seeds attract wireworms. A few days later, remove the plastic and soil covering the bait and count the number of wireworm larvae found at each station. There are not specific recommendations as to how many traps per field should be placed, however, placement of the bait stations should represent different areas of a field (Campbell & Stone, 1939; Simmons et al., 1998)

4.1.6 Control

There are no effective natural enemies for wireworm. If one suspects wireworms are present in a field based on trapping, chemical control is the best management option (http://potatoes.com/Research-IPM.cfm). Fumigants are effective on wireworms that are present at the time of fumigation and within the zone of fumigation (Schreiber et al., 2010). Fumigants are sensitive to soil temperatures. In furrow applications are also effective; however, some rotational restrictions may apply (Schreiber et al., 2010). Use of contemporary chemicals in other crops suggests that stand protection and wireworm reduction are not covered with current chemicals available (Vernon et al., 2009).

4.2 Colorado Potato Beetle (Order Coleoptera: Family Chrysomelidae)

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), first described in 1824 by Thomas Say, is associated with potato plants and its solanaceous relatives such as nightshade. It is the most important defoliating insect pest of potato. Its remarkable ability to develop insecticide resistance, incredible reproductive potential and sustained feeding by larvae and adults, makes the management of this pest challenging (Hoy et al., 2008).

4.2.1 Pest description

The Colorado Potato Beetle (CPB) is a yellow and black striped beetle, about 1.3 cm long and 0.6 cm wide. They can be found in almost all U.S. potato regions. Larvae are reddish orange, with two rows of black spots on each side. Orange egg clusters are found mainly on the undersides of leaves, mostly in the top third of the plant. Eggs resemble ladybug eggs (Fig. 6).

Fig. 6. Colorado potato beetle egg mass (left), larva (center), and adult (right). Photos by R. Marchosky (egg mass), L. Ketchum (larva), and S.I. Rondon (adult), OSU.

4.2.2 Damage
This beetle can cause complete defoliation and nearly complete crop loss if allowed to reproduce unchecked. Both larvae and adults feed on potato foliage throughout the season.

4.2.3 Hosts
Potatoes and other solanaceous plants such as eggplant, nightshade, horsenettle and buffalobur are preferred hosts of this pest.

4.2.4 Biology
Pupation and overwintering occur in the soil. Adults emerge from the soil to lay eggs in the spring. Depending on the region, this insect may have three generations in a season. Adult beetles spend the winter buried 10-25 cm in the soil and emerge in the spring just as the first volunteer potatoes appear. Recently emerged beetles either mate close to the overwintering sites or fly to new potato fields to find a mate. Usually first infestations occur around field margins. Eggs are deposited on potato foliage in masses. CPB eggs resemble lady beetle eggs. Larvae pass through four life stages and then burrow into the soil to pupate.

4.2.5 Monitoring
Start monitoring fields at crop emergence. There are no established treatment thresholds for CPB. Large CPB populations are harder to manage than small ones, thus the goal is to control this pest early in the season.

4.2.6 Control
Crop rotation may help in delaying or reducing CPB pressure. Colonizing beetles need to feed before laying eggs, so controlling volunteer potatoes and solanaceous weeds is important as are rotating crops and planting new potato fields far from the last year's potato fields (Schreiber et al., 2010). These practices will reduce the number of overwintering beetles migrating into the new field. This may not be a practical solution in the Pacific Northwest region since potatoes are use in rotation with other local crops such as wheat or corn. The use of "at planting" and systemic insecticides in early potatoes will contribute to the control of early-season CPB populations. The use of pyrethroid insecticides is not recommended since it has a direct effect on natural enemies. Targeting chemical applications to control eggs and young larvae when possible is recommended.

4.3 Green peach aphid and potato aphid (Order Heteroptera: Family Aphididae)
The aphid population in western North America, north of Mexico, is comprised of 1,020 species in 178 genera in 15 subfamilies (Pike et al., 2003). Several aphid species are known to be pests of potatoes, but the green peach aphid, *Myzus persicae* (Sulzer), and potato aphid, *Macrosiphum euphorbiae* (Thomas), are two of the most important vectors of diseases in the Pacific Northwest. Aphids are important due to their ability to transmit viruses. According to Hoy et al., (2008) there are six commonly found potato viruses transmitted by aphids: *Potato leafroll virus* (PLRV), multiple strains of *Potato virus Y* (PVY), *Potato virus A* (PVA), *Potato virus S* (PVS), *Potato virus M* (PVM), and *alfalfa mosaic virus* (AMV). PLRV and PVY are transmitted by several species of aphids but primarily by green peach aphid. The potato aphid transmits PVY and PVA.

4.3.1 Pest description

Green peach aphids are small, usually less than 0.3 cm long. The body varies in color from pink to green with three darker stripes down the back. The head has long antennae which have an inward pointing projection or tubercle at its base (Fig. 7). Potato aphids are larger than green peach aphids with a body somewhat elongated and wedge-shaped (Fig. 8). The adults of both species may be winged (alatae) or wingless (apterous). Winged forms are usually triggered by environmental changes (e.g., decreasing photoperiod or temperature, deterioration of the host plant or overcrowding) (Branson et al., 1966). On the back of the fifth abdominal segment, a pair of tube-like structures called "siphunculi", "cornicles", or "pipes" are present on most aphid species. The green peach aphid present a "swollen" cornicles with a dark tip, while the cornicles on the potato aphid are 1/3 of the length of the body and are usually curved slightly outward (Alvarez et al., 2003).

Fig. 7. Green peach aphid wingless adult (left) and alatae (right). Photos by A. Jensen, Washington Potato Commission.

Fig. 8. Potato aphid wingless adult and nymphs (left) and alatae (right). Photos by A. Jensen, Washington Potato Commission.

4.3.2 Damage

In general, aphids injure plants directly by removing sap juices from phloem tissues. They also reduce the aesthetic quality of infested plants by secreting a sugary liquid called "honeydew" on which a black-colored fungus called "sooty mold" grows. The "sooty mold" reduces the photosynthetic potential of the plant. Most importantly, aphids transmit plant diseases, particularly viruses. Aphids on potato are serious pests because of their ability to transmit several plant diseases such as PLRV (transmitted mainly by green peach aphid) and PVY (transmitted by several species of aphids). PLRV causes necrosis while strains of PVY can cause internal brown lesions in the tubers. Srinivasan & Alvarez (2007) reported that mixed viral infections of heterologous viruses occur regularly in potatoes.

4.3.3 Hosts

The green peach aphid, also known as tobacco or spinach aphid, survives the winter in the egg stage on peach trees. They can also overwinter on various perennial, biennial, and winter annual weeds, such as tumble mustard, flixweed, shepherd's-purse, chickweed, mallow, horseweed, pennycress and redstem filaree. Besides potatoes and peaches, other hosts include lettuce, spinach, tomatoes, other vegetables and ornamentals (Dickson & Laird, 1967; Wallis, 1967; Tamaki et al., 1980; Barry et al., 1982).

4.3.4 Biology

Green peach aphid migrates to potatoes in the spring from weeds and various crops where it has overwintered as nymphs and adults, or from peach and related trees where it overwinters as eggs. Most aphids reproduce sexually and develop through gradual metamorphosis (overwintering diapause egg, nymphs and winged or wingless adults) but also through a process called 'parthenogenesis' in which the production of offspring occurs without mating (Jensen et al., 2011). Potato aphids also overwinter as active nymphs, adults or eggs; eggs are laid on roses and sometimes other plants. Throughout the growing season aphids produce live young, all of which are female and can be either winged or wingless. In some instances, aphids undergo sexual, oviparous reproduction as a response of a change in photoperiod and temperature, or perhaps a lower food quantity or quality, where females produce sexual females and males. In the fall, winged males are produced which fly to overwintering hosts and mate with the egg-laying females produced on that host. Aphids found in the region undergo multiple overlapping generations per year (Jensen et al., 2011, Schreiber et al., 2010).

4.3.5 Monitoring

Fields should be checked for aphids at least once a week starting after emergence. The most effective scouting method is beating sheets, trays, buckets or white paper. There are no well-established treatment thresholds for aphids in potatoes in the Pacific Northwest but since aphids transmit viruses, producers are encouraged to control aphids early in the season, especially in seed potato producing areas. Schreiber et al., (2010) recommend a minimum sample size of ten locations per 100 acre field. For potatoes that are not to be stored, application of foliar aphidicide should begin when 5 aphids per 100 leaves or 5 aphids/plant are detected. Hoy et al., (2008) suggests some sampling methods and action thresholds for colonizing aphids on processing potatoes, table stock, and seed potato in different productions thresholds.

4.3.6 Control

Weed control and elimination of secondary hosts are critical. Early aphid infestations commonly occur on a number of weeds including species of mustards and nightshade; therefore, those weeds should be kept under control. Research in Idaho indicates that hairy nightshade is an excellent aphid and virus host (Srinivasan & Alvarez, 2007), thus, control of this weed is highly recommended. In some instances, the number of insects available to infest crops in the spring depends upon winter survival (DeBano et al., 2010). Thus, the elimination of overwintering sites is recommended if possible. Peach trees are the most common winter hosts, although apricots and several species of Prunus are sometimes infested (Schreiber et al., 2010). A large numbers of generalist predators feed on aphids including the minute pirate bugs, big-eyed bugs, damsel bugs, lady beetles and their larvae, lacewings, flower fly larvae, and aphid-specific parasitoid wasps. If aphids are present, use of insecticides in commercial fields should occur as soon as non-winged aphids are detected. In seed producing areas, preventive methods are recommended. Application of foliar aphidicide should begin just prior to the decline in performance of seed-treatment insecticides applied at planting (60 days after planting, Rondon unpublished). Schreiber et al., 2010 indicated that complete insect control from planting until aphid flights have ceased is the only means to manage diseases in full season potatoes.

4.4 Beet leafhopper (Order Heteroptera: Family Cicadellidae)

The beet leafhopper, *Circulifer tenellus* Baker, is the carrier of the beet leafhopper-transmitted virescence agent (BLTVA) phytoplasma (a.k.a., Columbia Basin potato purple top phytoplasma) that causes significant yield losses and a reduction in potato tuber quality.

4.4.1 Pest description

The beet leafhopper (BLH) is a wedge-shaped and pale green to gray or brown in color. It has several nymphal instars (Fig. 9). Adults may have dark markings on the upper surface of the body early and late in the season (”darker form”) or clear during the season (“clear form”) (Fig. 10).

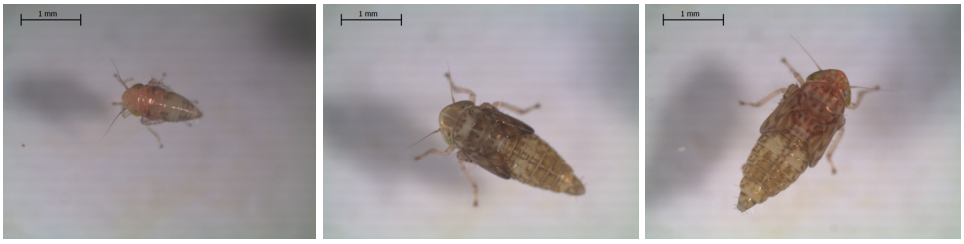

Fig. 9. Beet leafhopper nymphs. Photos by A. Murphy, OSU.

4.4.2 Damage

Beet leafhoppers must feed in the phloem of the plant. Direct feeding can cause relatively minor damage (“hopperburn”); however, BLTVA is a very destructive and detrimental disease affecting potatoes. BLTVA can cause a wide range of symptoms in potatoes, including leaf curling and purpling, aerial tubers, chlorosis, and early senescence. Most BLTVA infection occurs early in the season, during May and June (Munyaneza, 2003; Munyaneza & Crosslin,

2006). Potato is not a preferred host for BLH and will not spend much time on the crop (however it does spend enough time to transmit BLTVA) (Schreiber et al., 2010).

4.4.3 Hosts

Among the favorite hosts are Kochia, Russian thistle, and various weedy mustard species such as tumble mustard. Beet leafhoppers are especially abundant on young, marginal, semi-dry and small weeds plants. They also thrive on radishes, sugar beet (Meyerdirk & Hessein, 1985), and carrots (Munyaneza, 2003).

4.4.4 Biology

The beet leafhopper overwinters on rangeland weeds and migrates to potatoes as early as May. They overwinter as adult females in weedy and native vegetation throughout most of the dry production areas. The beet leafhopper has three life stages: egg, nymph and adult. The adult can have a "darker form", early or late in the season; and a "clear form (during the season) (Fig. 10). Beet leafhoppers can transmit BLTVA as adults and nymphs. Eggs are laid in stems of host plants, and a new spring generation begins developing in March and April. Beet leafhopper begins to move from weeds to potatoes and potentially affect potatoes during the first spring generation, which matures in late May to early June (Jensen et al., 2011). Potatoes are most seriously affected by BLTVA infections that occur early in the growing season (Rondon unpublished). Beet leafhopper remains common through the summer, during which it goes through 2 to 3 overlapping generations. The final generation for the year matures during late October-early November. Total number of beet leafhoppers varies from year to year (Crosslin et al., 2011).

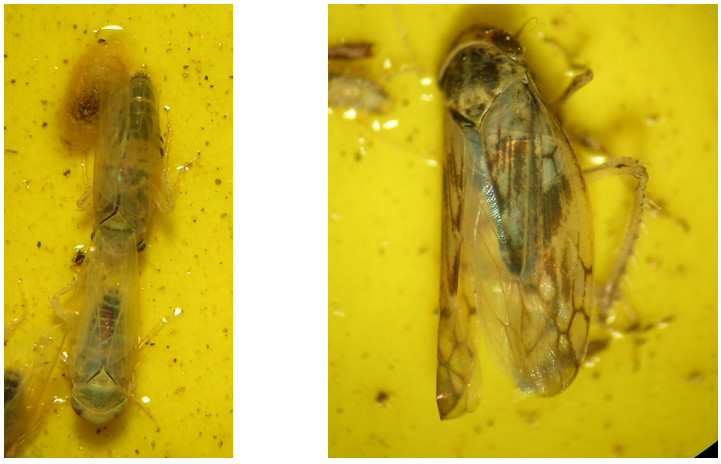

Fig. 10. "Clear form"(left) and "dark form" (right) of the beet leafhopper. Size of adults 2.5-3 mm. Photos by A. Jensen, Washington Potato Commission.

4.4.5 Monitoring

Because potatoes are not a preferred host of the BLH, in-field sampling is problematic. Most recommendations suggest the use of yellow sticky cards around field margins. It is important to keep traps close to the ground where hoppers mostly move. Check and replace

traps at least once a week. Rondon (unpublished data) suggests the use of DVAC (modified leaf blowers) to collect leafhoppers.

4.4.6 Control

Weed control in areas surrounding the potato field can help reduce initial sources of BLTVA inoculum. Due to the nature of the pest, few biological control efforts have been taking place in the Pacific Northwest. However, a species of *Anagrus* (Hymenoptera Mymaridae), has been reported as a common egg parasitoid in California (Meyerdirk & Moratorio, 1987).
Foliar insecticides can reduce BLH populations and ergo, the incidence of the disease. Based on extensive research conducted in the Pacific Northwest, there are several foliar applied insecticides that are effective against BLH. Some evidence suggests that the use of some neonicotinoid insecticides at planting may provide control of BLTVA (Schreiber et al., 2010).

4.5 Potato Tuberworm (Order Lepidoptera: Family Gelechiidae)

The potato tuberworm, *Phthorimaea operculella* Zeller, is one of the most economically significant insect pests of cultivated potatoes worldwide. The first significant economic damage to potato crops in the Columbia Basin region occurred in 2002, when a field in Oregon showed high levels of tuber damage associated with potato tuberworm. By 2003, the pest was a major concern to all producers in the region after potatoes from several fields were rejected by processors because of tuber damage. Since then, potato tuberworm has cost growers in the Columbia Basin millions of dollars through increased pesticide application and unmarketable potatoes (Rondon, 2010).

4.5.1 Pest description

The potato tuberworm has four life stages: adult, egg, larva and pupa. Adults are small moths (approximately 0.94 cm long) with a wingspan of 1.27 cm. Forewings have dark spots (2-3 dots on males; "X" on females). Both pairs of wings have fringed edges (Rondon & Xue, 2010) (Fig. 11). Eggs are ≤ 0.1 cm spherical, translucent, and range in color from white or yellowish to light brown. Eggs are laid on foliage, soil and plant debris, or exposed tubers (Rondon et al., 2007); however, foliage is the preferred oviposition substrate (Varela, 1988). Adult female moths lays 150-200 eggs on the underside of leaves, on stems, and in tubers (Hoy et al., 2008). Larvae are usually light brown with a characteristic brown head. Mature larvae (approximately 0.94 cm long) may have a pink or greenish color (Fig. 12). Larvae close to pupation drop from infested foliage to the ground and may burrow into the tuber. Ultimately, larvae will spin silk cocoons and pupate on the soil surface or in debris under the plant.

Fig. 11. Forewings of potato tuberworm adult females present an "x" pattern (left); while male (right) present 2-3 dark spots. Photos by OSU (Rondon 2010).

Fig. 12. Potato tuberworm larva entering tuber. Photo by L. Ketchum, OSU.

4.5.2 Damage

Tuberworm larvae behave as leaf miners. They can also live inside stems or within groups of leaves tied together with silk. The most important damage is to tubers, also a food source for the larvae, especially exposed tubers, or those within centimeters of the soil surface. Larvae can infest tubers when foliage is vine killed or desiccated right before harvest (Clough et al, 2010). Tunnels left by tuber worms in tubers can be full of droppings or excrement that can be a potential source for secondary infections.

4.5.3 Hosts

Although the potato tuberworm host range includes a wide array of Solanaceous crops such as tomatoes, peppers, eggplants, tobacco, and weeds such as nightshade, the pest has been found only on potatoes in the Pacific Northwest region (Rondon, 2010).

4.5.4 Biology

Potato tuberworm adults emerge as early as April in the Pacific Northwest, and continue to threaten the crop through November. Populations build sharply later in the growing season (September and October). The potato tuberworm has been detected in all potato growing regions of Oregon and throughout the Columbia Basin of Washington. A limited number of adults have been trapped in western Idaho. No tuber damage has been reported in Idaho (Rondon, 2010). A recent study suggests that locations with higher spring, summer, or fall temperatures are associated with increased trapping rates in most seasons (DeBano et al., 2010). Occasionally potato tuberworm pupae can be found on the surface of tubers, most commonly associated with indentations around the tuber eyes, but usually are not found inside tubers (Rondon et al., 2007). Considering the duration period of each instar and its relationship to abiotic factors such as temperature, the potato tuberworm can undergo several generations per year in the Pacific Northwest region.

4.5.5 Monitoring

Pheromone-baited traps to catch adult male moths have been widely used in the region (Rondon et al., 2007). Unfortunately there are no established treatment thresholds. Another

way is to check leaf mining. Most mines are found in the upper third of the plant canopy, suggesting that efficient scouting for foliar damage should focus on the top third of the plant (DeBano et al., 2010). The number of mines gives a good indication of the history of potato tuberworm infestation in a plant, but it does not necessarily indicate the severity of larval infestation at a point in time. The study also found that reasonably precise estimates of foliar damage for areas of 23 ft x 30 ft can be made by sampling 9 plants (DeBano et al., 2010).

4.5.6 Control

Control efforts should be directed toward tuberworm populations right before or at harvest. Females prefer to lay eggs on potato foliage, but when potato foliage starts to degrade and change color, or when it is vine-killed, the risk of tuber infestation increases greatly. The greatest risk for tuber infestation occurs between desiccation and harvest (Clough et al., 2010; Rondon, 2010). If tuberworm populations appear to be building prior to late season, additional control measures may be necessary. Other means of control include the elimination of cull piles and the elimination of volunteer potatoes. Daily irrigation that keeps the soil surface moist can also aid in the control of tuberworm populations. Most chemical products aim to reduce larva population in foliage but that technique does not provide 100% protection for the tubers.

4.6 Occasional pests

4.6.1 Mites

The two-spotted spider mite, *Tetranichus urticae* Koch, is the most abundant mite species found in potatoes in the Pacific Northwest. They can occasionally be considered pests of potatoes when crops such as beans, corn, alfalfa or clover seed are planted nearby (Hoy et al., 2008). Mites in general prefer hot and dry conditions; they also prefer stressed plants where irrigation is poorly managed. They damage plants by puncturing the leaf tissue to extract plant juices. Plants respond by changing color from green to brown. Spider mites overwinter in the area as adults in debris around field edges (Jensen et al., 2011). Females are very prolific; after emerging from overwinter, they mate and lay eggs on the underside of leaves. If temperatures are warm (75-80^{o}F or 23.8-26.6C), eggs can hatch in 3-5 days; nymphs to adults can take place in 7-9 days at those temperatures. When leaves get overcrowded, mites climb to the top of the plant and secrete silk that can be used as a "transport" device during light to moderate winds conditions (Fig. 13).

Sampling for mites requires a close visual inspection of leaves from different levels of the plants. Shaking potentially infested leaves above a piece of white paper helps to determine the presence of mites. Applications of miticides should be made upon early detection of mites. All potatoes should be surveyed for the presence of mites and mite eggs starting mid-season (Schreiber et al., 2010). Thorough coverage is essential for good control and it is suggested that foliage should be dry at the time of application. While a single application of a miticide will suffice, if a second application of a miticide is required, the use of a miticide with different chemistry should be considered as a resistance prevention strategy (Jensen et al., 2011).

4.6.2 Cutworm, armyworm and loopers

These are several species of moth larvae that affect potato crops. Cutworms, armyworms and loopers are the immature stages of lepidopteran moths. Moths' typically have four defined life stages: egg, larva, pupa and adult. The most common species in the Pacific

Fig. 13 Two spotted spider mite adults range in size from 0.25 mm to 0.5 mm long; eggs are around 0.1 mm. Adults and nymphs are pale yellow or light green with two dark spots on the abdomen (Photo by R.E. Berry, OSU).

Northwest regions are listed below (Table 2). Cutworms feed on potato seeds, cut stems, and foliage; armyworms and loopers feed on foliage throughout the season. Cutworms and armyworms have three pairs of true legs and five pairs of prolegs behind; loopers have only three pair of true legs and three pair of prolegs behind. At planting insecticides protect potato seed from cutworms; however, after the residual effect is gone, the crop is unprotected; in some years, a foliar chemical application may be needed. Potatoes can tolerate some worm defoliation without loss in marketable yield. The period of full bloom is the most sensitive plant growth stage, but even then defoliation on the order of 10% appears to cause little if any yield loss. Applications should be targeted to control small larvae (1st and 2nd instars), rather than larger larvae (Schreiber et al., 2010, Jensen et al., 2011).

Group	Common name	Scientific name
Cutworms	Spotted cutworms	*Xestia c-nigrum*
Western yellow striped armyworm	Bertha armyworm	*Mamestra configurata* Walker
Looper	Alfalfa looper	*Autographa californica* (Speyer)
	Cabbage looper	*Trichoplusia ni* (Hübner)

Table 2. Most common cutworm, armyworm, looper species in the Pacific Northwest (Zack. et al., 2010).

4.7 Resistance to insecticides

Insecticides are the most powerful tool available for use in pest management (Metcalf, 1994). However the misuse, overuse and historically unnecessary use of insecticides have been some of the most important factors in the increasing interest in integrated pest management (Von Rumker & Horay, 1972; Metcalf, 1994). In the last decades, the Insecticide Resistance Action Committee (IRAC), a group of technical experts that coordinates responses to prevent or delay the development of resistance in insect and mite pests, defined resistance to insecticides as a "heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used

according to the label recommendations for that pest species" (http://www.irac-online.org/). In other words, it is the inherited ability of a pest population to survive a pesticide which is a result of a process of selection (Hamm et al., 2008). Some potato pests have developed resistance to certain groups of pesticides; however, significant insecticide resistance is not yet known to occur in the Pacific Northwest (University of California, 1986). For instance, while spider mite infesting potatoes has demonstrated the ability to readily develop resistance to miticides there appears to be no evidence of this problem developing in the U.S. Pacific region (Schreiber et al., 2010).

Pesticides such as pyrethroids that disturb natural enemies can cause a resurgence of primary or secondary pests, especially when applied mid to late season. In the past few years, package mixes of insecticide, some including pyrethroids have been available for use on potatoes. More research is needed to evaluate the real impact of this pesticide in the Pacific Northwest potato region. Seed and soil treatments with systemic insecticides have become a standard approach to control early "invaders" (Hoy et al., 2008). This approach may be less disruptive to predator and non-target insects than traditional foliar or ground chemical applications.

There are several key components to developing a resistance management program for insect pests: first, producers must employ non-chemical control tactics for control of pest problems, including irrigation, cultivation and proper fertilization management; second, producers must rotate insecticidal modes of action. This integrated pest management approach will lead producers to a sustainable production system with long term economic benefits. Alvarez et al., (2003) suggest keeping good records of chemical applications, rotating insecticide use changing not only the product but also the class of compound, applying insecticides at labeled rates, using newer insecticides with chemistries that are safer for applicators and non-target organisms, and reducing insecticide applications by scouting and making applications only as needed.

5. Conclusions

Potato is one of the most important food crops widely grown over many latitudes and elevations over the world. Increasing potato production in a sustainable manner requires an integrated approach covering a range of strategies. Combating pests is a continuous challenge that producers have to face as they intensify their production techniques to satisfy the increasing demands of the global market.

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7. References

Alvarez, J.M., R.L. Stotlz, C.R. Baird, and L.E. Sandoval. (2003). Insect pest and their management. In *Potato Production System*s (ed) J.C. Stark and S.L. Love. University of Idaho Extension. Pp 205-239.

Andrews N., M. Ambrosino, G. Fisher, and S.I. Rondon. (2008). Wireworm biology and non-chemical management in potatoes in the Pacific Northwest. *Oregon State University Extension Service Publication*. Dec. PNW 607. Available at http://extension.oregonstate.edu/catalog/pdf/pnw/pnw607.pdf.

Barry, A., R.E. Berry, and G. Tamaki. (1982). Host preference of the green peach aphid, *Myzus persicae* (Hemiptera: Aphididae). *Environmental Entomology*. 11(4): 824-827.

Branson, T., F. Terry, and G. Robert. (1966). Effects of a nitrogen deficient host and crowding on the corn leaf aphid. *Journal of Economic Entomology*. 59(2): 290-294.

Cambell, R.E. & M.W Stone. (1939). Trapping elaterid beetles as a control measure against wireowmrs. *Journal of Economic Entomology*. 32: 47-53.

CIP (Centro Internacional de la Papa or International Potato Center). (1994). *International Potato Center Circular* 20(1). CIP, Lima, Perú.

Clough, G.H., S.I. Rondon, S.J. DeBano, N. David, and P.B. Hamm. (2010). Reducing tuber damage by the potato tuberworm (Lepidoptera: Gelechiidae) with cultural practices and insecticides. *Journal of Economic Entomology* 103(4): 1306-1311.

Crosslin, J.M., S.I. Rondon and P.B. Hamm. (2011). Population dynamics of the beet leafhopper in northern Oregon and incidence of the Columbia Basin potato purple top phytoplasma. American Journal of Potato Research (In press).

DeBano, S.J., P.B. Hamm, A. Jensen, S.I. Rondon, and P.J. Landolt. (2010). Spatial and temporal dynamics of potato tuberworm (Lepidoptera: Gelechiidae) in the Columbia Basin of the Pacific Northwest. *Journal of Economic Entomology* 39(1): 1-14.

Dickson, R., & E.F. Laird. (1967). Fall dispersal of green peach aphids to desert valleys. *Annals of the Entomological Society of America*. 60(5): 1088-1091.

Douches, D.S, W. Li, K. Zarka, J. Coombs, W. Pett, E. Grafius, T. El-Nasr. (2002). Development of Bt-cry5 insect-resistant potato lines 'Spunta-G2' and 'Spunta-G3'. HortScience 37: 1103-1107.

Dreistadt, S.H., J.K. Clark and M.L. Flint. (2004). *Pests of Landscape Trees and Shrubs: An Integrated Pest Management Guide*. 2nd edition, Publication 3359. University of California Division of Agriculture and Natural Resources, Davis. Pp 501.

Driesche, R.G., & T.S. Bellows. (1996). *Biological control*. Chapman and Hall. International Thomson Publishing Company. New York. Pp 539.

Ferro, D. N. (1994). Biological control of the Colorado potato beetle. *In*: G. W. Zehnder, M. L. Powelson, R. K. Jansson and K. V. Raman (eds), *Advances in Potato Pest Biology and Management*. APS Press, St. Paul. Pp. 357-375.

Gopal, J. & S.M.P. Khurana. (2006). Potato production, improvement and postharvest management. The Haworth press. Pp 587.

Hamm, P.B.., C,W. Hy, P.J. Hutchinson, W.R. Stevenson, R.A. Boydston, J.M. Alvarez, A. Alyokin, G. Dively, N.C. Gudmestad, and W.W. Kirk. (2008). Managing pesticide resistance. *In Potato Health Management. Plant Health Management Series* (Ed. D.A. Johnson). American Phytopathological Society, St Paul, Minnesota, USA. Second Edition. Pp 123-131.

Hawkes, J.G. (1990). *The potato, evolution, biodiversity, and genetic resources*. Belhaven Press, London. Pp 259.

Heitefuss, R. (1989). *Crop and plant protection: the practical foundations*. Ellis Horwood Ltd, Chichester. Pp 261.

Hoy, C.W., G. Boiteau, A. Alyokhin, G. Dively, and J.M. Alvarez. (2008). Managing insect and mite pests. In (Ed D. Johnson) *Potato Health Management. Plant Health*

Management Series. American Phytopathological Society, St Paul, Minnesota, USA. Second Edition. Pp 133-147.

Horgan, F.G., D.T. Quiring, A. Lagnaoui, A. Salas, and Y. Pelletier. (2007). Mechanism of resistance to tuber-feeding *Phthorimaea operculella* (Zeller) in two wild potato species. *Entomological Experimental Applicata* 125: 249-258.

Horton, D. (2006). Quantitative relationship between potato tuber damage and counts of Pacific coast wireworm (Coleoptera: Elateridae) in baits: seasonal effects. *Journal of the Entomological Society of British Columbia* 103: 37-48.

Hoy, C.W., G. Boiteau, A. Alyokhin, G. Dively, J.M. Alvarez. (2008). Managing insects and mites. *In Potato Health Management. Plant Health Management Series* (Ed. D.A. Johnson). American Phytopathological Society, St Paul, Minnesota, USA. Second Edition. Pp 133-147.

Hutchinson, C.M., & W.M. Stall. (2007). *Potato vine killing or desiccation*. University of Florida IFAS Extension. HS925. Available at http://edis.ifas.ufl.edu/pdffiles/HS/HS18100.pdf.

Jensen, A. , A. Schreiber and N. Bell. (2011). Irish potato pests. In Pacific Northwest Insect Management Handbook. Ed. C. Holligsworth. U. Massachusetts Extension. Available at http://uspest.org/pnw/insects?00INTR05.dat.

Johnson, D.A. (2008). *Potato Health Management.* Plant Health Management Series. American Phytopathological Society, St Paul, Minnesota, USA. Second Edition. Pp 259.

Koss, A. (2003). *Integrating chemical and biological control in Washington State potato fields*. M.S. Thesis, Washington State University, Pullman, WA, USA.

Lacey, L.A., D.R. Horton, T.R. Unruh, K. Pike, and M. Marquez. (2001). Biological control of insect pests of potato in north america. *In Washington State Potato Conference and Trade Show proceedings*. Spanish seminar. Tri Cities, WA. Pp 123

Lopez, R., & D.N. Ferro. (1995). Larviposition of Myiopharus doryphorae (Diptera: Tachinidae) to Colorado potato beetle (Coleoptera: Chrysomelidae) larvae treated with lethal and sublethal doses of *Bacillus thuringensis* Berliner subsp. tenebrionis. *Journal of Economic Entomology* 88(4); 870-874.

Love, S.L., P. Nolte, D.L. Corsini, J.C. Whitmore, L.L. Ewing, and J.L. Witworth. 2003. Seed production and certification. In *Potato production systems*. (ed) J.C. Stark and S.L. Love. University of Idaho Extension. Pp 49

Luckmann, W.H., & R.L. Metcalf. &. (1994). Pest management concept. *In Introduction to Insect Pest Management*. 3rd edition. A Wiley-Interscience publication. John Wiley & Son. Pp 1-34.

Metcalf, R.L., & W.H. Luckmann. (1994). *Introduction to Insect Pest Management*. 3rd edition. A Wiley-Interscience publication. John Wiley & Son. Pp 650.

Metcalf, R.L. (1994). Insecticides in Pest Management. In *Introduction to Insect Pest Management* (ed R.L. Metcalf and W.H. Luckmann). 3rd edition. A Wiley-Inter science publication. John Wiley & Son. Pp 245-314.

Merrill, S.C., S.M. Walter, F.B. Peairs, and J.A. Hoeting. (2011). Spatial variability of western bean cutworm (Lepidoptera: Noctuidae) pheromone trap captures in sprinkler irrigated corn in eastern Colorado. *Environmental Entomology* 40(3): 654-660.

Meyerdirk, D.E. & N.A. Hessein. (1985). Population dynamics of the beet leafhopper, *Circulifer tenellus* (Baker), and associated *Empoasca* spp. (Homoptera: Cicadellidae) and their egg parasitoids on sugar beets in southern California. *Journal of Economic Entomology*. 78: 346-353.

Meyerdirk, D.E. & M.S. Moratorio. (1987). Biology of *Anagrus giraulti* (Hymenoptera: Mymaridae), and egg parasitod of the beet leafhopper, *Circulifer tenellus* (Homoptera: Cicadellidae). *Annals of the Entomological Society of America.* 80: 272-277.

Miller, J.S., & B.G. Hopkins. (2008). Checklist for a holistic potato health management plan. In *Potato Health Management. Plant Health Management Series* (Ed. D.A. Johnson). American Phytopathological Society, St Paul, Minnesota, USA. Second Edition. Pp 7-10.

Ministry of Agriculture. (2008). *Native potatoes of Perú*. J & J Proyects Editoriales S.A.C. Pp 115.

Munyaneza, J.E. (2003). Leafhopper identification and biology. In *Pacific Northwest Vegetable Association Proceedings*. Pp 89-91.

Munyaneza, J.E., and J.M. Crosslin. (2006). The beet leafhopper (Hemiptera: Cicadellidae) transmits the Columbia basin potato purple top phytoplasma to potatoes, beets, and weeds. *Journal of Economic Entomology*. 99(2): 268-272.

Pedigo, L.P. 1996. *Entomology and Pest Management*. Second Edition. 1996. Prentice-Hall Pub., Englewood Cliffs, NJ. Pp 679.

Persoons, C.J., S. Voerman, P.E.J. Verwiel, F.J. Ritter, W.J. Nooyen, and A.K. Minks. (1976). Sex-pheromone of potato tuberworm moth, *Phthorimaea operculella:* isolation, identification and field evaluation. *Entomological Experimental Applicata.* 20: 289-300.

Pike, K.S., L.L. Boysdston, and D.W. Allison. (2003). *Aphids of western North America north of Mexico*. Washington State University Extension. MISC0523. Pp 282.

Raman, K.V. (1988). Control of potato tuber moth *Phthorimaea operculella* with sex pheromones in Perú. *Agriculture, Ecosystems and Environment* 21: 85-99.

Roelofs, W.L., J.P. Kochansky, R.T. Carde, G.G. Kennedy, C.A. Henrick, J.N. Labovitz, and V.L. Corbin. (1975). Sex-pheromone of potato tuberworm moth, *Phthorimaea operculella*. *Life Sciences* 17: 699-706.

Rondon, S.I., S.J. DeBano, G.H. Clough, P.B. Hamm, A. Jensen, A. Schreiber, J.M. Alvarez, M. Thornton, J. Barbour, and M. Dŏgramaci. (2007). Biology and management of the potato tuberworm in the Pacific Northwest. *Oregon State University Extension Service Publication*. Apr. PNW 594.
http://extension.oregonstate.edu/catalog/pdf/pnw/pnw594.pdf.

Rondon, S.I., D. Hane, C.R. Brown, M.I. Vales, and M. Dŏgramaci. (2009). Resistance of potato germplasm to the potato tuberworm (Lepidoptera: Gelechiidae). *Journal of Economic Entomology*. 102(4): 1649-1653.

Rondon, S. I., S. J. DeBano, G. H. Clough, P. H. Hamm, and A. Jensen. (2008). Ocurrence of the potato tuber moth in the Columbia Basin of Oregon and Washington. J. Kroschel and L. Lacey (eds) *Integrated pest management for the potato tuber moth, Phthorimaea operculella Zeller: a potato pest of global importance*. Tropical Agriculture 20, Advances in Crop Research 10 Margraf Publishers, Weikersheim, Germany: 9-13.

Rondon, S.I. (2010). The potato tuberworm: a literature review of its biology, ecology, and control. *American Journal of Potato Research.* 87:149–166.

Rondon, S.I. & L. Xue. (2010). Practical techniques and accuracy for sexing the potato tuberworm, *Phthorimaea operculella* (Lepidoptera: Gelechiidae). *Florida Entomologist*. 93(1): 113-115.

Rothschild, G.H.L. (1986). The potato moth: an adaptable pest of short term cropping systems. In: Kitching R. L. (Ed.), *The ecology of exotic plants and animals*. J. Wiley, Brisbane. Pp 144-162.

Salaman, R. (1985). *The history and social influence of the potato*. Cambridge University Press. Pp 685.

Schreiber, A., A. Jensen, K. Pike, J. Alvarez, and S.I. Rondon. (2010). *Integrated Pest Management guidelines for insects and mites in Idaho, Oregon, and Washington Potatoes*. http://oregonstate.edu/potatoes/ipm/publications.htm.

Simmons, C., L. Pedigo, and M.E. Rice. (1998). Evaluation of seven sampling techniques for wireworms. *Environmental Entomology* 27(5): 1062-1068.

Srinivasan, R. & J.M. Alvarez. (2007). Effect of mixed viral infections (potato virus Y-potato leafroll virus) on biology and preference of vectors *Myzus persicae* and *Macrosiphum euphorbiae* (Hemiptera: Aphididae). *Journal of Economic Entomology*. 100(3): 646-655.

Tabashnick, B.E. (1989). Managing resistance with multiple pesticides tactics: theory, evidence, and recommendations. *Journal of Economic Entomology*. 92: 1263-1269.

Tamaki, G., L. Fox, and R.L. Chauvin. (1980). Green peach aphid: orchard weeds are host to fundatrix. *Environmental Entomology*. 9(1): 62-65.

Tamaki, G., R.L. Chauvin and A.K. Burditt, Jr. (1983). Field evaluation of *Doryphorophaga doryphorae* (Diptera: Tachinidae), a parasite, and its host the Colorado potato beetle (Coleoptera: Chrysomelidae). *Environmental Entomology*. 12: 386-389.

University of California. (1986). *Integrated Pest Management for potatoes in the western United States*. University of California, Division of Agriculture and natural Resources. Publication 3316. Pp 146.

Varela, L.G., and E.A. Bernays. (1988). Behavior of newly hatched potato tuber moth larvae, *Phthorimaea operculella* Zell. (Lepidoptera: Gelechiidae), in relation to their host plants. *Journal of Insect Behavior*. 1: 261-275.

Vernon, R.S., W.G. van Herk, M. Clodius, and C. Harding. (2009). Wireworm management I: Stand protection versus wireworm mortality with wheat seed treatments. *Journal of Economic Entomology* 102(6): 2126-2136.

Voerman, S., and G.H.L. Rothschild. (1978). Synthesis of 2 components of sex-pheromone system of potato tuberworm moth, *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae) and field experience with Them. *Journal of Chemical Ecology*. 4: 531-542.

Von Rumker, R. & F. Horay. (1972). Pesticide manual. *U.S. Agency for International Development*. Pp. 126.

Wale, S., H.W. Platt, and N. Cattlin. (2008). *Potatoes a color handbook*. Manson publishing, Ltd. London. Pp

Wallis, R.L. (1967). Some host plants of the green peach aphid and beet western yellow virus in the Pacific Northwest. *Journal of Economic Entomology*. 60(4): 904-907.

Washington Potato Commission. (2007). *Potato Varieties in the Northwest*. (Ed.) A. Jensen. Vol VII, No15. Available at www.potatoes.com

Weisz, P.R., Z. Smilowitz, M.C. Saunders, and B. Christ. (1995). *Integrated pest management for potatoes*. Pennsylvania State Unviersity Cooperative Extension, Unviersity Park, PA. Pp 63.

Weinsz, P.R., S. Fleischer, and Z. Smilowitz. (1996). Site-specific integrated pest management for high value crops: impact on potato pests management. *Journal of Economic Entomology*. 59(2) 501-509.

Zack, R.S., P.J. Landolt, A. Jensen, and A.Schreiber. (2010). Lepidopterous "worms" on, but not in potatoes. In *Annual progress report-Washington Potato Commission*. Pp 1-5. Available at www.potatoes.com.

15

Management of *Tuta absoluta* (Lepidoptera, Gelechiidae) with Insecticides on Tomatoes

Mohamed Braham and Lobna Hajji
Centre régional de recherche en Horticulture et Agriculture Biologique; Laboratoire d'Entomologie – Ecologie; Chott-Mariem, Tunisia

1. Introduction

Tomato, *Lycopersicon esculentum* Mill is a vegetable crop of large importance throughout the world. Its annual production accounts for 107 million metric tons with fresh market tomato representing 72 % of the total (FAO, 2002). It is the first horticultural crop in Tunisia with a production area of 25,000 hectares and a total harvest of 1.1 million metric tons (DGPA, 2009) of which nearly 70 % are processed (Tomatonews, 2011). Tomatoes are grown both under plastic covered greenhouses and in open field.
The tomato leafminer, *Tuta absoluta* Meyrick, (Lepidoptera : Gelechiidae) is a serious pest of both outdoor and greenhouse tomatoes. The insect deposits eggs usually on the underside of leaves, stems and to a lesser extent on fruits (photo 1). After hatching, young larvae penetrate into tomato fruits (photo 2), leaves (photo 3) on which they feed and develop creating mines and galleries. On leaves, larvae feed only on mesophyll leaving the epidermis intact (OEPP, 2005). Tomato plants can be attacked at any developmental stage, from seedlings to mature stage.

Photo 1. *T. absoluta* egg

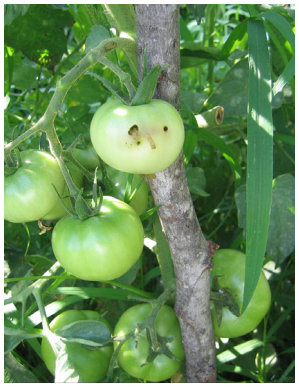

Photo 2. Larvae on fruit

Photo 3. Larva of *T. absoluta*

Originated from South America, *T. absoluta* was reported since the early 1980s from Argentina, Brazil and Bolivia (Estay, 2000); the insect rapidly invaded many European and

Mediterranean countries. It was first recorded from eastern Spain in late 2006 (Urbaneja, 2007), then Morocco, Algeria, France, Greece, Malta, Egypt and other countries (for a complete list see www.tutaabsoluta.com; Roditakis *et al*., 2010, Mohammed, 2010).
Chemical control using synthetic insecticides is the primary method to manage the pest, but it has serious drawbacks, including reduced profits from high insecticide costs, destruction of natural enemy populations (Campbell *et al*., 1991), build-up of insecticide residues on tomato fruits (Walgenbach *et al*., 1991) and in the environment and fundamentally the rapid development of insecticide resistance. For example, resistance development has been reported against abamectin, cartap, methamidophos and permethrin in Brazil (Siqueira *et al*., 2000a, Siqueira *et al*., 2000b) and against deltamethrin and abamectin in Argentina (Lietti *et al*., 2005). Thus, in order to avoid selection of resistant biotypes, a careful management with frequent changes of active ingredients is desirable. Furthermore, modern integrated pest management recommends effective pesticides that have low mammalian toxicity, low persistence in the environment and high degree of selectivity. Since insecticide control currently remains an indispensable tool, the goal is to minimize the amount and impact of pesticides through the diversification of active ingredients used.
In this paper, we present the data from insecticides trials conducted in 2009 and 2010 under laboratory and field conditions, in which the efficacy of several hitherto untested insecticides and natural products was compared with that the widely used insecticides to manage *T. absoluta* in Tunisia such as spinosad, indoxacarb and pyrethroids compounds.

2. Material and methods

2.1 Laboratory trials

2.1.1 Laboratory assays in 2009

Tomato seeds (cv Topsun) were sown on 30 January 2009. Seeds were deposited in 110 cm3 cells in a rectangular polyester tray of 60 cm x 40 cm x 5 cm filled with peat (Potgrond H, Germany). On March 3, 2009, seedlings were transplanted into 1 liter plastic flowerpot (bottom diameter =8 cm, top diameter = 12 cm and height = 12 cm) filled with peat without fertilization and watered as required. The tomato plants were maintained in the laboratory until use. Three days before the assay, plants (having four to six true leaves) were deposited in a tomato crop situated in the vicinity of the laboratory to permit *T. absoluta* egg-laying then transferred to the laboratory. Leaves were examined under binocular microscope and *T. absoluta* larvae were counted. Insecticides were sprayed using a hand sprayer (1 liter of capacity). After drying, the treated plants were kept in an unsealed empty greenhouse bordering the laboratory. There were four replications (plants) for each product and an untreated plant was used as a check. The efficacies of the products were tested twice: 48 hours following sprays and 12 days later. The Insecticides and natural plant extracts used are given in table 1.

2.1.2 Laboratory assays in 2010

A colony of *T. absoluta* was established from larvae and pupae collected from tomato infested field in the Chott-Mariem region. The insect was reared and maintained in a small greenhouse (10*6 m). From time to time, tomato leaves harboring *T. absoluta* pre-imaginal stages collected in the field were introduced in the rearing greenhouse.
Tomato seeds (cultivar Riogrande) were sown on February 13, 2010 in a rectangular polyester tray as mentioned before. Plants having four to six true leaves were transferred to the rearing greenhouse and remained there for 2 to 3 days to allow egg-laying. Thereafter

Active ingredients	Trade name	Companies	Dose cc/ hl water
deltamethrin	Decis EC25	Bayer Crop Science	100 cc/hl
bifenthrin	Talstar	FMC Corporation	100 cc/hl
acetamiprid	Mospilan 200 SL	Basf	50 cc/hl
methomyl	Lannate 25	Dupont de Nemours	150 cc/hl
metamidophos	Tamaron 40	Bayer Crop Science	150 cc/hl
abamectin	Vertimec	Syngenta	30 cc/hl
Spinosad	Tracer	Dow-Agroscience	60 cc/hl
Rotenone	Rotargan	Atlantica Agricola (Spain)	300 cc/hl
Neem extract	Oleargan	Atlantica Agricola	100 cc/hl

Table 1. Insecticides and natural plant extracts used in the laboratory trial in 2009.

returned to the laboratory and put in wooden cages for insecticide trials. Leaves were examined under binocular microscope and *T. absoluta* larvae were counted just before insecticide spray (April 3, 2010) and regularly after 2 to 3 days post-treatment. Dead larvae following trial were recorded. The second insecticide spray was done on April 19, 2010 (two weeks later). The Insecticides and natural plant extracts used are given in table 2.

Active ingredients	Trade name	Companies	Dose cc/ hl water
diafenthiuron	Pegasus	Syngenta	125 cc/hl
triflumuron	Alystin SC 480	Bayer Crop science	50cc/hl
emamectin benzoate	Proclaim®	Syngenta	2500 grams/hl
Plant extracts	Tutafort	AltincoAgro (Spain)	125 cc/hl

Table 2. Insecticides and natural plant extracts used in the laboratory in 2010.

2.2 Field trials

2.2.1 Trials using natural products

Field experiments using botanical extracts, Spinosad and Kaolin Clay were conducted from March 2010 to May 2010 in a half commercial tomato greenhouse (34 meters long x 8 meters width) in Saheline region, Tunisia (35°42' North, 10°40'East). Tomato seeds (cv Sahel) were sown on 27 October 2009 in an expanded polyester tray under plastic protected nursery bed. Four double rows of tomato were transplanted on 23 November 2009. The plot (greenhouse) was prepared according to usual cropping practices in the region. Ploughing, tillage and second tillage to incorporate manure, bed formation, irrigation device establishment and drip irrigation.

Active ingredients	Trade name	Companies	Dose cc/ hl water
Spinosad	Tracer 240	Dow- Agroscience	60 cc/hl
Neem extract	Oleargan	Atlantica Agricola (Spain)	100 cc/hl
Kaolin Clay	Surround WP™	Engelhard Corporation (NJ.U.S.A)	5 kg/hl
Orange extract	Prev-am™	ORO Agri International Ltd	300 cc/hl
Botanical extracts	Deffort	AltincoAgro (Spain)	350 cc/hl
Botanical extracts	Armorex	Soil Technologies Corp (U.S.A)	60 cc/hl
Botanical extracts (*Quassia amara* and Neem)	Conflic	Atlantica Agricola (Spain)	250 cc/hl

Table 3. Natural products experimented in 2010.

Plots measured 4 m2 each (10 plants) arranged in a randomized block design with four replications. The active ingredients, the trade name and doses of the natural products are given in table 3. The products were diluted with tap water and applied at field rates based on the recommended label dilutions without surfactants.

2.2.2 Trials using insecticides

Trials using insecticides were undertaken during the same period in the second half greenhouse. Plot measured 8 square meters each (20 plants) arranged in a randomized block design with four replications. Three chemical compounds were used (table 4).

Active ingredients	Trade name	Companies	Dose cc/ hl water
indoxacarb	Avaunt 150EC	Dupont	50 cc/hl
triflumuron	Alsystin SC 480	Bayer Crop science	50 cc/hl
diafenthiuron	Pegasus 500SC	Syngenta	125 cc/hl

Table 4. Insecticides compounds experimented under tomato greenhouse in 2010.

Insect monitoring

To assess the *T. absoluta* infestation prior to the trial, thirty leaf samples, taken from about 30 different plants were weekly collected (from January to March 2010) at random from the entire greenhouse. The sample was placed in a plastic bag and taken to the laboratory. Leaves were examined under binocular microscope (Leica MZ12.5); eggs, larvae pupae, of *T. absoluta* live or dead as well as mines were recorded. However, only larvae (live or dead) were presented in this study.

2.3 Statistical analysis

Data on the effectiveness of various insecticides were analyzed using the Minitab Software for Windows (Minitab 13.0). The mean number of live larvae per plant or per leaf was tested for Normality assumption by Kolmogorov-Smirnov test then the data were square root transformed. General linear model procedures were used to perform the analysis of

variance. Wherever significant difference occurred, Tukey's multiple comparison test was applied for mean separation.

In the laboratory trial of 2010, due to the low number of live larvae in the control, a one way-ANOVA percentage of mortality was used instead of corrected mortality.

The percentages of efficacies of insecticides were evaluated either:

i. Abbott formula : the percentage of efficacy = (Ca-Ta)/Ca*100 where Ca is the average live larvae in the control and Ta is the mean survival score in the treatment.
ii. The percentage of larval mortality = mean number of dead larvae/(mean number of dead larvae + mean number of live larvae)*100.

3. Results

3.1 Laboratory trials

3.1.1 Assays in 2009

One day before the assay, the mean number of total live larvae (L1 to L4 instars) per plant varied from 0.75 to 3. There is no significant difference between treatments (GLM-ANOVA. F= 0.99, df= 9,30; P = 0.47, table 5). Three days after the first application, the mean number of live larvae per plant decreases in all treatments except in the control (Table 5). All insecticides significantly reduced *T. absoluta* larvae when compared with non treated control (F= 4.24, df = 9,30; P= 0.001, Table 5). However, the level of suppression by acetamiprid and bifenthrin did not differ significantly from the control (Table 5).

Mean number of larvae/plant on indicated days before treatment (DBF) and days after treatment (DAT)					
Insecticides !	1DBT1!!	3DAT1	5DAT1	8DAT1	12DAT1
spinosad(1)	1.75a	0.5a(86.66)*	0.50a(85.71)*	0.5a (87.5)*	0.25a(93.75)*
neem extract(2)	1.5a	0.75a(80)	0.75a(78.50)	0.5a(87.5)	0.5a(87.5)
rotenone(3)	0.75a	0.25a(93.33)	0.25a(92.90)	0.5a(87.5)	0.75a(81.25)
deltamethrin(4)	0.5a	0a(100)	1a(71.42)	0.75a(81.25)	1.5ab(62.5)
acetamiprid(5)	2a	1.25ab(66.66)	1.25ab(64.28)	1.25ab(68.75)	0.50a(87.5)
methomyl(6)	3a	0.5a(87)	0.5a(86)	0.50a(88)	0.75a(81)
metamidophos(7)	2a	0.75a(80)	0.75a(79)	0.75a(81)	1.00a(75)
abamectin(8)	2.25a	0.75a(80)	0.75a(79)	0.5a(88)	0.25a(94)
bifenthrin(9)	2a	1.25ab(67)	2ab(43)	1.25ab(69)	1.00a(75)
Control	2.5a	3.75b	3.5b	4b	4b
Statistical analysis	F= 0.99	F= 4.24	F= 3.69	F= 4.20	F= 4.66
ANOVA-	df = 9,30	df = 9,30	df = 9,30	df = 9,30	df = 9,30
GLM	P = 0.47	P= 0.001	P= 0.003	P= 0.001	P=0.003

! denote commercial compounds: (1): Tracer, (2): Oleargan, (3): Rotargan, (4): Decis, (5): Mospilan, (6): Lannate (7): Tamaran, (8): Vertimec, (9): Talstar

!! Means followed by the same letter within a column are not significantly different at P= 0.05 (ANOVA-GLM procedure) followed by Tukey multiple comparison

* Data in brackets denote percent Abbott mortality (Abbott, 1925)

Table 5. Mean number of *T. absoluta* total live larvae/plant on indicated days before treatment (DBF) and days after treatment (DAT) (the first treatment was done on April 1, 2009).

Five days following the first application, all the products performed well except acetamiprid and bifenthrin which show no significant difference compared with the control (Table 5). Eight days after the first application, the mean number of total live larvae per plant varied from 0.5 to 4. All the tested products reduced significantly the density of live larvae per plant compared with the control (F= 4.20; df = 9,30; P= 0.001). Still, acetamiprid and bifenthrin showed mild efficacy (table 5). At 12 days following treatments, all the products performed well (F= 4.66, df = 9,30 ; P= 0.003), yet the plants treated with deltamethrin show increasing mean live larvae per plant (table 5).
Regarding the corrected mortality according to Abbott formula, Spinosad and rotenone gave satisfactory results post-treatment (88.4 % and 88.7% respectively) followed by Lannate (85%), Vertimec (85%), neem extract (83. 22), and Tamaran (79%). However, Decis (78.8%), Mospilan (71.8) and Talstar (63%) showed mild efficacy. Though, Decis performed well till 8 days following the first application (84.2%).

Mean number of total live larvae/plant on indicated days after the second treatment (DAT)				
Insecticides	0DBT2!!	2DAT2	4DAT2	8DAT2
spinosad	0a	0a(100)*	0a(100)*	0.75a(83.33)*
neem extract	0.5a	0.5a(92.85)	0.75a(78.60)	1.75a(61.11)
rotenone	0.25a	0.5a(85.71)	0.5a(85.71)	1.25a(72.22)
deltamethrin	0.75a	0.75a(78.60)	1a(71.42)	1.25a(72.22)
acetamiprid	0.5a	0.5a(85.71)	0.75a(78.60)	1.5a(66.66)
methomyl	1.25a	0.75a(78.60)	0.75a(78.60)	2a(83.33)
metamidophos	0.75a	0.75a(78.60)	0.5a(85.71)	1a(77.71)
abamectin	0.5a	0.5a(85.71)	0.5a(85.71)	1.5a(66.66)
bifenthrin	1a	1a(64.28)	1.5ab(57.14)	2a(55.55)
Control	3.5b	3.5b	3.5b	4.5b
Statistical analysis	F= 6.07	F= 7.24	F=5.84	F= 4.39
	df = 9,30	df = 9,30	df = 9,30	df = 9,30
	P = 0.00	P= 0.00	P= 0.00	P= 0.001

* Data in brackets denote percent Abbott mortality (Abbott, 1925)
!! : Means followed by the same letter within a column are not significantly different at P= 0.05 (ANOVA-GLM procedure) followed by Tukey multiple comparison

Table 6. Mean number of total *T. absoluta* live larvae/plant the day of the second treatment and thereafter (DAT2) (the treatment was undertaken on April 21)

Just before the second application, the mean number of live larvae in treated plants remained low compared with the control. It varied between zero (Tracer) and 3.5 (control) (table 6). Two days following the second insecticide application, all tested compounds show good efficacy compared with control (F=4.24; df = 9,30; P<0.001). Spinosad (Tracer) performed well (100 % efficacy according to Abbott corrected mortality formula). However, bifenthrin (Talstar) shows mild efficacy (table 6). The same conclusion can be formulated four days following treatments (table 6). At eight days after trial, the insecticide spinosad remains active and performed well (83.33 % efficacy) (table 6).
The overall efficacy according to Abbott formula (1925) shows the good performance of spinosad (Tracer), rotenone (Rotargan), methomyl (Lannate), abamectin (Vertimec) (Fig. 1.).

However, the percentage of larval mortality (number of dead larvae/sum of dead and live larvae) following the first and second insecticide application shows the best performance of spinosad (91 %), neem extract (71 %) and abamectin (71%).

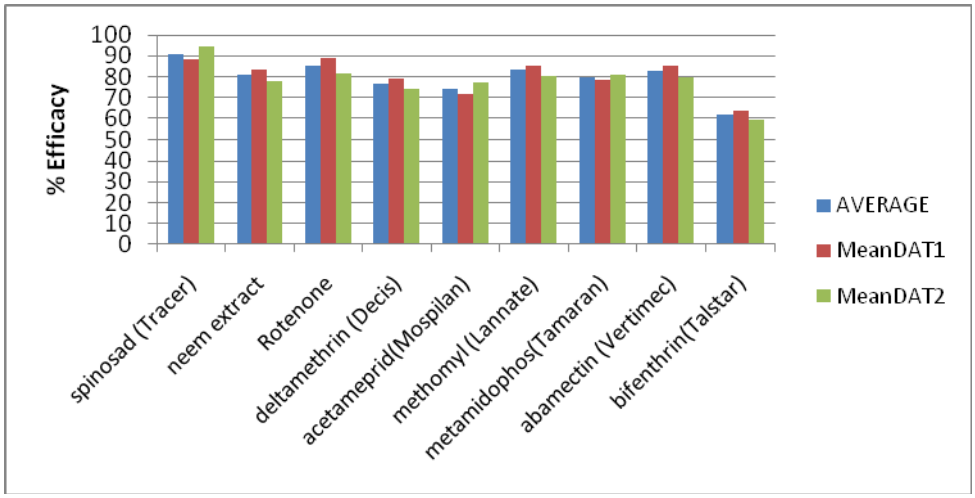

Fig. 1. Overall percentage of efficacy according to Abbott formula (1925). DAT1 = days after the first treatment, DAT2 = days after the second treatment (laboratory trial, 2009).

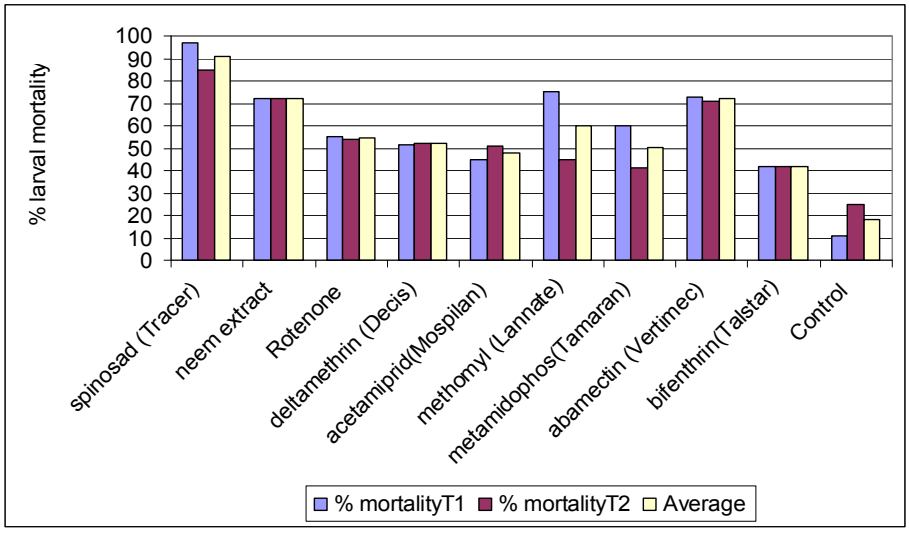

Fig. 2. Percentage of larval morality following the first (T1) and the second treatment (T2) (mean number of four dates after the fist treatment and 3 dates after the second treatment).

3.1.2 Assays in 2010

Just before the first spray (April 3, 2010), the mean number of live larvae (first to fourth instars) per leaf varied from 0.12 (Control) to 0.52 (Proclaim®). Although there is no significant difference between treatments (ANOVA-GLM F= 1.37, df = 4, 116; P=0.24), the control plants harboured less live larvae (table 7). There is no larval mortality.

Two days following the first spray (April 5), there is no significant difference between treatments regarding live larvae (GLM; F= 0.93, df = 4, 116; P= 0.46. Table 7). However, the percentage of larval mortality did vary (ANOVA, 1 factor, F = 4.17; df = 4, 120; P= 0.003) showing the best performance of Proclaim® (57.14 %; Table 7).

Nine days after the first insecticide application (April 12), the mean number of live larvae per leaf did not significantly vary between treatments (ANOVA-GLM procedure Table 7). However, the percentage of mortality significantly varies between treated and untreated plants (ANOVA 1 factor, F= 3.07; df = 4, 120; P= 0.021). The maximum percentage of mortality is given by Proclaim® (45.70%, table 7).

At 11 days after the first insecticide application (on April 14), the mean number of live larvae did not significantly vary among treated and untreated plants (ANOVA - GLM procedures Table 7). However, the percentage of mortality did vary according to treatments (F = 3.16, df = 4, 120; P= 0.017) showing the good efficacy of Proclaim® (52.93 % Table 7).

Mean number of live larvae/leaf on indicated days before treatment (DBF) and days after treatment (DAT)µ					
Insecticides!	0DBT! !	2DAT1	9DAT1	11DAT1	13DAT1
(1)	0.36(0)a	0.36(10)a	0.37(13.61)a	0.44(12.66)a	0.34(12.82)a
(2)	0.32(0)a	0.2(37.5)a	0.24(29.47)a	0.34(23.52)a	0.34(20.05)a
(3)	0.52(0)a	0.24(57.14)a	0.29(45.70)a	0.25(52.93)a	0.23(51.51)a
(4)	0.44(0)a	0.48(0)a	0.26(17.91)a	0.20(21.91)a	0.18(27.39)a
(5)	0.12(0)a	0.24(0)a	0.22(0)a	0.25(0)a	0.20(0)a
Statistical analysis	F= 1.37	F=0.90	F= 0.57	F=0.63	F=0.27
ANOVA	df =4,116	df =4,116	df =4,116	df =4,116	df =4,116
-GLM	P =0.24	P =0.46	P = 0.67	P=0.64	P= 0.89

! :(1):triflumuron(Alystin), (2) plant extract (Tutafort), (3) emamectin benzoate (Proclaim®) (4) diafenthiuron (Pegasus) and (5) Control.

µ: Data under brackets denote percentage of mortality

! ! : Means followed by the same letter within a column are not significantly different at P= 0.05 (ANOVA-GLM procedure) followed by Tukey multiple comparison

Table 7. Mean number of live *T. absoluta* larvae on indicated days before treatments and days after treatments (laboratory trial, 2010)

At 13 days after the first application, the mean number of live larvae did not significantly vary between treatments and control (Table 7). However, the percentage of mortality significantly varies between treated and control plants (F = 3.53 df = 4, 120; P= 0.009) showing the good efficacy of the compound Proclaim® (51.51 %, table 7).

At 16DAT1 and just before the second spray, the mean number of live larvae shows no significant difference between treated and control plants (table 7. continued). However, the percentage of mortality did significantly vary between treated and control plants (One way

ANOVA F= 4.95 df = 4, 120; P= 0.001). The compound Proclaim® shows the highest mortality percentage (54.83 % table 7.Cont.).
At three days after the second insecticide application, there is no significant difference regarding the mean number of live larvae per leaf (GLM-ANOVA). Nevertheless, plants treated with the product Proclaim® harbour zero live larvae per leaf suggesting the good efficacy of this insecticide. This is confirmed by the high percentage of mortality (100 %) as well as the significant difference between treated and control plants (One way ANOVA, F= 4.51 df = 4, 120; P= 0.002).

Mean number of live larvae/leaf on indicated days before treatment (DBF) and days after treatment (DAT)(µ)					
Insecticides	16DAT1!	3DAT2	5DAT2	8DAT2	10DAT2
(1)	0.33(12.55)a	0.19(51.71)a	0.16(59.91)ac	0.15(59.4)ac	0.15(59.14)ac
(2)	0.31(21.47)a	0.06(80.15)a	0.06(83.64)ac	0.06(83.35)ac	0.06(83.35)ac
(3)	0.20(54.83)a	0(100)a	0(100)bc	0(100)bc	0(100)bc
(4)	0.20(19.60)a	0.16(0)a	0.1(59.55)ac	0.09(59)ac	0.09(59)ac
(5)	0.20(0)a	0.16(0)a	0.16(0)a	0.16(0)a	0.06(0)a
Statistical analysis	F= 0.27	F= 2.02	F= 1.85	F= 1.85	F= 1.56
	df =4, 116	df =4, 116	df =4, 116	df =4, 116	df =4, 116
GLM-ANOVA	P= 0.89	P= 0.096	P= 0.123	P= 0.096	P=0.189

µ : Data under brackets denote percentage of mortality
! : Means followed by the same letter within a column are not significantly different at P= 0.05 (ANOVA-GLM procedure) followed by Tukey multiple comparison

Table 7. (continued). Mean number of live *T. absoluta* larvae on indicated days before treatments and days after treatments (laboratory trial, 2010)

Five days after the second spray, the mean number of live larvae did not vary among treated and untreated plants (table 7. Cont.). But the percentage of mortality significantly varies (ANOVA one factor F= 3.98 df = 4, 120; P= 0.03) showing again the good performance of Proclaim® (table 7.Cont.).
At eight days after the second spray, there is no significant difference between treated plants and control regarding the mean number of live larvae (table 7.Cont.). However, the percentage of mortality varies (ANOVA, one factor, F= 3.88 df = 4, 120; P= 0.005). The compounds Proclaim® and Tutafort are the best (100 % and 83.35 respectively, table 7.Cont.).
At 10 days after the second insecticide application, there is no significant difference between treated plants and control (GLM-ANOVA, Table 7.Cont.). Concerning the percentage of mortality, there is a significant difference between treated and control plants (ANOVA, one factor, F= 3.99 df = 4, 120; P= 0.006). Proclaim® followed by Tutafort performed well (100 % and 83.35 respectively, table 7.Cont.).

3.2 Field trials

3.2.1 Natural products experimented in 2010 under greenhouse

The first spray was undertaken on March 26, 2010, then on April 8 and on April 19, 2010.
At three days following the first application, the mean live larvae (small and old larvae) per leaf did not significantly vary between treated and control plots (GLM-ANOVA Procedure,

P= 0.09). Although, plots treated with spinosad show the minimum live larvae as demonstrated by 70% efficacy according to Abbott formula (Table 8). The details of larval instars (small larvae: first and second instars and old larvae: three and fourth instars) show a significant difference between insecticides tested. The compounds Tracer, Armorex and Deffort performed well (table 9).

Mean number of total larvae/leaf on indicated days before treatment (DBF) and days after treatment (DAT)					
Insecticides	1DBT! !	3 DAT1*	10DAT1(μ)	2DAT2	6DAT2
Armorex(1)	0.30a	0.20(20)a	0.1(69.23)a	0(100)a	0.325(0)a
Deffort(1)	0.30a	0.25(0)a	0.45(0)a	0.475(0)b	0.3(0)a
Oleargan (1)	0.20a	0.32(0)a	0.225(30.76)a	0.05(33.33)a	0.25(0)a
Konflic(1)	050a	0.57(0)a	0.2(38.46)a	0.125(0)a	0.075(0)a
Prev-am™ (2)	0.32a	0.37(0)a	0.45(0)a	0.1(0)a	0.2(0)a
Surround WP™ (3)	0.30a	0.32(0)a	0.25(23.07)a	0.075(0)a	0.15(0)a
Tracer(4)	0.1a	0.075(70)a	0.05(84.61)a	0(100)a	0.025(0)a
Control	0.20a	0.25a	0.325a	0.075a	0.025a
Statistical Analysis	F= 1.42	F= 1.94	F= 1.61	F= 1.61	F= 1.92
	df =3, 309	df =3, 309	df =3, 309	df =3, 309	df =3, 309
GLM- ANOVA	P= 0.120	P= 0.09	P= 0.131	P=0.008	P=0.066

(1): Botanical extracts
(2): Orange extract
(3) : Kaolin
* Corrected mortality according to Abbott formula
μ = second spray
! ! : Means followed by the same letter within a column are not significantly different at P= 0.05 (ANOVA-GLM procedure) followed by Tukey multiple comparison

Table 8. Mean number of total live larvae following natural products applications under tomato greenhouse (Saheline, Tunisia, 2010).

At 10 days after the first natural products applications, the ANOVA-GLM procedure shows no significant difference between treatments regarding the mean number live larvae (Table 8). The Abbott's percentages of efficacy show the performance of spinosad (84.61 %) and the plant extract (Armorex; 69.23%).
At two days after the second spray, (April 10) there is a significant difference between treated plots (AVOVA-GLM procedure, P= 0.008, table 8). The plots treated with Deffort show the maximum density of mean live larvae per leaf (table 8). However, there is no significant difference between the other products and control. The details of larval stages confirm the low efficacy of Deffort compared with the other products and control (small larvae : P= 0.026; Old larvae P= 0.019; table 9).
Six days following the second application (April 14), the mean number of live larvae shows no significant difference between treated and untreated plots (Table 8).
At eleven days after the second spray, the mean number of live larvae per leaf is relatively similar among treatments and did not significantly vary (ANOVA-GLM procedure P= 0.211) varying from 0.1 to 0.9. Plots treated with Kaolin (Surround) harbour the minimum density.
Four days after the third spray (April 23, 2010), the treated plot differed significantly showing the good performance of the compounds neem extract, Tracer and Konflic (table 8). This is confirmed by the analysis of detailed larval instars (table 9).

At nine days after the third spray, the mean number of total larvae varied between 0.2 and 2.05. The ANOVA-GLM procedure showed a significant difference between treatments. The products Tracer, Armorex and Deffort were effective in reducing *T. absoluta* larval densities (table 8).

Mean number of total larvae/plant on indicated days before treatment (DBF) and days after treatment (DAT)				
Insecticides	11 DAT2!	4DAT3! !	9DAT3	18DAT3
Armorex(1)	0.525(0)a	0.1(85.18)a	0.3(85.36)b	0.9(12.2)a
Deffort(1)	0 .925(0)a	0.3(55.55)a	0.2(90.24)b	0.65(36.85)a
Oleargan (1)	0.325(13.33)a	0.075(88.88)ab	0.55(73.17)b	0.375(63.4)a
Konflic(1)	0.475(0)a	0(100)ab	0.825(59.75)a	0.325(68.3)a
Prev-am™ (2)	0.225(40) a	0.175(74.07)a	1.675(18.30)a	0.7(31.7)a
Surround(3)	(3)0.1(73.33)a	0.2(70.37)a	0.55(73.17)b	0.375(63.4)a
Tracer(4)	0.35(6.66)a	0.1(85.18)a	0.25(87.80)b	0.75(26.8)a
Control	0.375a	0.675a	2.05a	1.025a
Statistical Analysis	F=1.41	F=2.49	F=2.49	F=1.36
	df= 7,309	df= 7,309	df= 7,309	df= 7,309
GLM-ANOVA	P=0.201	P=0.017	P=0.000	P=0.220

! : third spray

! ! : Means followed by the same letter within a column are not significantly different at P= 0.05 (ANOVA-GLM procedure) followed by Tukey multiple comparison

Table 8. (Continued) Mean number of total live larvae following natural products applications under tomato greenhouse (Saheline, Tunisia, 2010).

Mean number of live larvae/leaf on indicated days before treatment (DBF) and days after treatment (DAT)				
Insecticides	3DAT1! !		10DAT1	
	SL*	OL*	SL*	OL*
Armorex(1)	0.075(40)a	0.125(0)a	0.1(50)a	0(100)a
Deffort(1)	0.05(60)a	0.2(0)a	0.225(0)a	0,225(0)a
Oleargan (1)	0.2(0)a	0.125(0)a	0.175(12.5)a	0.05(60)a
Konflic(1)	0.425(0)b	0.15(0)a	0.1(50)a	0.1(20)a
Prev-am™ (2)	0.25(0)ab	0.125(0)a	0.275(0)a	0.175(0)a
Surround WP™ (3)	0.3(0)a	0.025(80)a	0(100)a	0.25(0)a
Tracer(4)	O(100)a	0.075(40)a	0.05(75)a	0(100)a
Control	0.125(0)ab	0.125(0)a	0.2(0)a	0.125(0)a
Statistical Analysis	F= 4.03	F= 0.77	F= 1.76	F= 1.53
	df= 3,309	df= 3,309	df= 3,309	df= 3,309
GLM-ANOVA	P= 0.00	P= 0.611	P= 0.096	P=0.157

*: SL : Small larvae (L1-L2), OL: Old larvae (L3-L4)

! ! : Means followed by the same letter within a column are not significantly different at P= 0.05 (ANOVA-GLM procedure) followed by Tukey multiple comparison.

Table 9. Mean number of live small and old larvae following natural products applications under tomato greenhouse (Saheline, Tunisia, 2010).

Mean number of larvae/plant on indicated days before treatment (DBF) and days after treatment (DAT)				
Insecticides	2 DAT2! !		6DAT2	
	SL*	OL*	SL*µ	OL*
Armorex(1)	0(100)a	0(100)b	0.1b	0.225(0)a
Deffort(1)	0.175(0)b	0.3(0)a	0.025a	0.275(0)a
Oleargan (1)	0.025(0)a	0.025(50) b	0.175b	0.075(0)a
Konflic(1)	0.05(0)a	0.075(0)b	0a	0.075(0)a
Prev-am™ (2)	0(100)a	0.1(0)b	0.125b	0.075(0)a
Surround(3)	0.025(0)a	0.05(0)b	0.1b	0.05(0)a
Tracer(4)	0(100)a	0(100)b	0a	0.025(0)a
Control	0.025a	0.05b	0a	0.025a
Statistical analysis GLM-ANOVA	F= 2.31	F= 2.44	F= 2.18	F= 1.34
	df= 3,309	df= 3,309	df= 3,309	df= 3,309
	P= 0.026	P=0.019	P=0.036	P=0.069

µ: undetermined Abbott percentage of efficacy (zero Small larvae in the control plot)
SL: Small larvae (L1-L2), OL: Old larvae (L3-L4).
! ! : Means followed by the same letter within a column are not significantly different at P= 0.05 (ANOVA-GLM procedure) followed by Tukey multiple comparison

Table 9. (Continued) Mean number of live small and old larvae following natural products applications under tomato greenhouse (Saheline, Tunisia, 2010).

Mean number of larvae/plant on indicated days before treatment (DBF) and days after treatment (DAT)				
Insecticides	11 DAT2! !		4DAT3	
	SL*	OL*	SL*	OL*
Armorex(1)	0.375(0)a	0.15(0)a	0(100)a	0.1(84.61)ab
Deffort(1)	0.7(0)a	0.225(0)a	0.15(0)b	0.15(76.9)ab
Oleargan (1)	0.25(0)a	0.075(50)a	0.025(0) a	0.05(92.30)b
Konflic(1)	0.425(0)a	0.05(66.66)a	0(100)a	0(100)b
Prev-am™ (2)	0.075(66.66)a	0.15(0)a	0(100)a	0.175(73.0)b
Surround WP™ (3)	0.07(66.66)a	0.02(83.33)a	0.125(0)b	0.075(88.4)b
Tracer(4)	0.275(0)a	0.075(50)a	0.075(0)ab	0.02 (96.15)b
Control	0.225a	0.15a	0.025a	0.65a
Statistical Analysis	F=1.31	F=1.10	F=2.75	F=2.82
	df=3,309	df=3,309	df=3,309	df=3,309
GLM-ANOVA	P=0.246	P=0.361	P=0.009	P=0.007

*: SL : Small larvae (L1-L2), OL: Old larvae (L3-L4)
! ! : Means followed by the same letter within a column are not significantly different at P= 0.005 (ANOVA-GLM procedure) followed by Tukey multiple comparison

Table 9. (Continued) Mean number of live small and old larvae following natural products applications under tomato greenhouse (Saheline, Tunisia, 2010).

Mean number of larvae/plant on indicated days before treatment (DBF) and days after treatment (DAT)				
Insecticides	9 DAT3! !		18DAT3	
	SL*	OL*	SL*	OL*
Armorex(1)	0.125(72.22)a	0.175(89.06)b	0.75(0)a	0.15(75) a
Deffort(1)	0.025(94.44)b	0.175(89.06)b	0.4(5.88)a	0.25(58.33)a
Oleargan (1)	0.225(50)a	0.325(79.68)b	0.075(82.35)a	0.3(50)a
Konflic(1)	0.275(38.88)a	0.55(65.62)a	0.175(58.82)a	0.15(75) a
Prev-am™ (2)	0.375(16.78)a	1.3(18.75)a	0.45(0)a	0.25(58.33)a
Surround WP™ (3)	0.1(77.77)a	0.45(71.87)a	0.2a	0.175(70.83)a
Tracer(4)	0.125(72.22)a	0.125(92.18)b	0.45(0)a	0.3(50)a
Control	0.45a	1.6a	0.425a	0.6a
Statistical Analysis	F= 2.33	F=5.68	F=1.41	F= 1.97
	df = 3,309	df = 3,309	df = 3,309	df = 3,309
ANOVA-GLM	P= 0.00	P=0.000	P=0.201	P=0.06

* Data in brackets denote percent Abbott mortality (Abbott, 1925)
! !: Means followed by the same letter within a column are not significantly different at P= 0.05 (ANOVA-GLM procedure) followed by Tukey multiple comparison

Table 9. (Continued) Mean number of live small and old larvae following natural products applications under tomato greenhouse (Saheline, Tunisia, 2010).

Three days following the first insecticide application, the mean number of live larvae (small and large) did not vary significantly between treated and untreated plots (ANOVA-GLM Procedure F= 1.94, df = 3, 309 P= 0.063). However, the plants treated with spinosad (Tracer) harbor the minimal larval density (Table 9).

3.2.2 Insecticides compounds experimented under tomato greenhouse in 2010

Four days before the first insecticide application, the mean number of live larvae per leaf varied between 0.6 and 0.97 showing no significant difference between treatments and control (ANOVA. GLM, F= 0.82, df =3, 156; P=0.82).

Two days following the first treatment (March 24), the mean number of live larvae remains relatively low and did not significantly vary between treatment and control (F = 0.34; df = 3, 153; P= 0.79). The corrected mortality according to Abbott formula shows slight efficacy of tested products (Table 10).

At 12 days following the first application, the mean number of live larvae significantly differed between treatments (GLM, F=2.90, df = 3, 156; P= 0.037). The Tukey multiple comparisons showed the good performance of indoxacarb (Avaunt) (Table 10). There is no significant difference between plot treated with triflumuron (Alystin), diafenthiuron (Pegasus) and untreated plots.

Three days after the second treatment, there is a significant difference between treated plots and control (GLM, F= 16.45 df = 3, 153; P= 0.000). The three compounds performed well particularly Avaunt (92.30 % according to Abbott formula).

Nine days following the second spray, all insecticides performed well compared with the control (F= 46.7 df =3,153; P=0.000) with the best performance of indoxacarb (Avaunt) (96.87 % efficacy according to Abbott formula, Table 10).

Mean number of larvae/leaf on indicated days before treatment (DBF) and days after treatment (DAT)μ					
Insecticides	4DBT1! !	2 DAT1	12DAT1	3DAT2	9DAT2
indoxacarb	0.87a	0.7(15. 15)a!	0.2(71.42) a!	0.05(92 .30)a	0.075(96.87)a
triflumuron	0.97a	0.6(27.27)a	0.52 (25)ab	0.1(84.61)a	0.4(83.33)a
diafenthiuron	0.6a	0. 72 (12.12)a	0.4(42.85)ab	0.125(80.76)a	0.30(87.5)a
Control	0.87a	0.85a	0.7 b	0.65b	2.4b
Statistical analysis	F=0.82	F= 0.43	F=2.90	F= 16.45	F= 46.7
	df =3, 153	df = 3, 153	df = 3, 153	df =3, 153	df =3, 153
GLM-ANOVA	P=0.48	P=0.72	P=0.037	P=0.000	P=0.000

μ : the first treatment was undertaken on March 22, 2010.
! : data in brackets denote percentage of efficacy (Abbott Formula)
! ! : Means followed by the same letter within a column are not significantly different at P= 0.05 (ANOVA-GLM procedure) followed by Tukey multiple comparison

Table 10. Mean number of *T. absoluta* larvae/leaf on indicated days before treatment (DBF) and days after treatment (DAT) (Saheline tomato greenhouse, 2010).

Mean number of larvae/leaf on indicated days before treatment (DBF) and days after treatment (DAT)			
Insecticides	18DAT2! !	3DAT3	12DAT3
indoxacarb (Avaunt)	0.05(95.83)a	0.075(95.45)a	0.35(78.12)a
triflumuron (Alystin)	0.05((95.83)a	0.5(69.69)a	0.7(56.25)a
diafenthiuron (Pegasus)	0.075(93.75)a	0.325(80.30)a	0.32(87.5)a
Control	1.2b	1.65b	1.6b
Statistical analysis	F= 40.88	F= 20.91	F=10.87
	df =3, 153	df =3, 153	df =3, 153
	P = 0.00	P= 0.00	P= 0.000

! ! : Means followed by the same letter within a column are not significantly different at P= 0.05 (ANOVA-GLM procedure) followed by Tukey multiple comparison

Table 10 (continued). Mean number of *T. absoluta* larvae per leaf on indicated days before treatment (DBF) and days after treatment (DAT) (Saheline tomato greenhouse, 2010).

At 18 days following the second application, the mean number of live larvae significantly varies between treated and control plots (GLM F= 40.88; df = 3, 153; P= 0.000). The efficacy of tested insecticide remains high compared with the control.
At 3 and 12 days following the third insecticide application all tested insecticides continue to be effective compared with the control (F= 20.91 df =3, 153; P= 0.00 ; F=10.87; df =3, 153; P= 0.00). Nevertheless, indoxacarb (Avaunt) tend to be a powerful suppressor of *T. absoluta* larvae (table 10).

4. Discussion

In Argentina, the primary *T. absoluta* management tactic was chemical sprays (Lietti *et al.*, 2005). Organophosphates were initially used for *T. absoluta* control then were gradually replaced by pyrethroids during the 1970s. During the early 1980s, cartap which alternates with pyrethroids and thiocyclam were sprayed showing the good effectiveness of the former. During the 1990s, insecticides with novel mode of actions were introduced such as abamectin, acylurea, insect growth regulators, tenbufenozide and chlorfenapyr (Lietti *et al.*, 2005).
Our laboratory results demonstrate the efficacy of spinosad (Tracer), rotenone (Rotargan), methomyl (Lannate) and abamectin (Vertimec). Methomyl was only tried due to its highly used frequency in tomato production against Noctuid larvae in Tunisia.
Spinosad, a mixture of spinosyns A and D, is derived from the naturally occurring actionomycete, *Saccharopolyspora spinosa* (Sparks *et al.,* 1998). Because of its unique mode of action, involving the postsynaptic nicotinic acetylcholine and Gamma-aminobutyric (GABA) receptors, spinosad has strong insecticidal activity against insects (Salgado, 1998) especially Lepidoptera (e.g. *Helicoverpa armigera* (Wang *et a*l., 2009), *Spodoptera frugiperda* (Méndez *et al.*, 2002), Diptera (King and Hennesey 1996; Collier and Vanstynwyk , 2003 ; Bond *et al*., 2004), some Coleoptera (Elliott et al., 2007) as well as stored grains (Hertlein *et al*, 2011).
To date, spinosad is considered a good alternative control of Lepidopteran pests due to its high activity at low rates and its use in integrated pest management programs. The product possesses advantages in term of safety for farm workers and consumers due to its low mammalian toxicity and rapid breakdown in the environment (Sparks et al., 1998). The compound is considered as a standard product for the control of *T. absoluta* in Brazil (Maraus *et al.*, 2008) showing, however low efficacy compared with the insecticide novaluron.
Rotenone has been reported to be an excellent insecticide against a wide range of insect pests. Davidson (1930) found that rotenone was a toxic and effective contact insecticide against several species of whiteflies, aphids, caterpillars and mites. Also, Turner (1932) reported a high toxicity of rotenone to larvae of the Colorado potato beetle *Leptinotarsa decemlineata* (Say).
Azadirachtin, a tetranortriterpenoid isolated from the seeds of neem tree, *Azadirachta indica* (Meliaceae), and the fruit of chinaberry, *Melia azaderach* (Meliaceae) acts as an antifeedant and inhibits the growth and the development of several insects (Meisner *et al.*, 1981, Raffa, 1987; McMillian *et al*., 1969). The antifeefant effects of azadirachtin are partly due to sensory detection and avoidance by insects (Simmonds and Blaney 1984).
Acetamiprid (Mospilan) is a neonicotinoid insecticide that is formulated for both soil and foliar application. It is a broad-spectrum insecticide effective against several groups of

insects including Lepidopterans, Coleopterans, Hemipterans and Thysanopterans. The insecticide has an ingestion and stomach action and has a strong osmotic and systemic action (Takahashi *et al*., 1998). The compounds interact with Acetylcholine receptors (AChRs) in a structure-activity relationship, resulting in excitation and paralysis followed by death (Ishaaya *et al*., 2007).

Abamectin a mixture of avermectins is extracted by the fermentation of the soil bacterium *Streptomyces avermitilis* (Strong & Brown 1987). The insecticide acts on the GABA receptor activating the chloride channel (nerve and muscles) (Aliferis and Jabaji, 2011).

Throughout the assay, the product emamectin benzoate (Proclaim®) showed the best efficacy strongly suppressed *T. absoluta* larval populations. Indeed, several authors reported the performance of this product against several insects, for example, Seal (2005), reported the efficacy of emamectin benzoate at various rates in reducing the densities of the melon thrips, *Thrips palmi* adults and larvae. Stanley *et al.,* (2005) reported the high acute toxicity of emamectin benzoate to *Helicoverpa armigera* under laboratory conditions.

Cook et al., (2004) conducted field and laboratory trials on cotton and soybean for the control of the beet armyworm *Spodoptera exigua* (Hübner) and the fall armyworm *Spodoptera frugiperda* using indoxacarb, pyridalyl, spinosad methoxyfenozide and emamectin benzoate demonstrated the good efficacy of tested products compared with the control. Plots treated with indoxacarb, spinosad and emamectin benzoate had significantly fewer beet armyworm larvae.

Avermectins are a family of 16-membered macrocyclic lactone natural product homologues produced by the soil microorganisms, *Streptomyces avermitilis.* They act as agonists on GABA and glutamate gated chloride channels. The chloride ion flux produced by the direct opening of channels into neuronal cells results in loss cell function and disruption of nerve impulses. Consequently, arthropods are paralyzed irreversibly and stop feeding. Maximum mortality is achieved within four days (Jansson *et al.,* 1997).

Emamectin benzoate (Proclaim) is a novel semi-synthetic derivative of the natural product abamectin in the avermectin family. This insecticide has a high potency against a broad spectrum of lepidopterous pests with an efficacy of about 1,500-fold more potent against certain armyworm species (Jansson *et al.,* 1996)

Insect growth regulators like triflumuron, lufenuron are claimed to be safe and have little impact on beneficial arthropods compared with conventional insecticides and thus attracted considerable attention for their inclusion in IPM programs (Ishaaya *et al*., 2007). In this study, triflumuron showed low efficacy against *T. absoluta* larvae. These results are in accordance with data reported by El-Sheikh and Aamir (2011) suggesting the greater efficiency of lufenuron in controlling *Spodoptera littoralis* Boisd compared with triflumuron or flufenoxuron. Similarly, low effectiveness of triflumuron (Alystin SC48) for the control of *Cactoblastis cactorum* (Lepidoptera: Pyralidae) was reported in Argentina by Labos et al., (2002). Yet the concentration used was lower (30 cc/ hl). Regarding the control of the Mediterranean fruitfly, *Ceratitis capitata*, triflumuron (Alystin 25) failed to give satisfactory results (a concentration of 150 ppm did not kill adults, Zapata *et al.,* (2006)).

Diafenthiuron (Pegasus) is a new type of thiourea derivative that affects respiration in insects. It disrupts oxidative phosphorylation by inhibition of the mitochondrial ATP synthase, an enzyme with essential role in cellular bioenergetics (Ishaaya, 2010). It is an insecticide and acaricide which kills larvae, nymphs and adults by contact and/or stomach action, showing also some ovicidal action (e-pesticide manual, 2005). In our laboratory trial, diafenthiuron (Pegasus) shows little efficacy in *T. absoluta* larval suppression (table 10).

Tutafort (plant extract) shows little efficacy after the first application but increases effectiveness after the second application engendering about 80 % of larval mortality (table 7.Cont.). Yet according to manufacturer, (Altinco, 2011), the product has a preventive action and should be applied against eggs and adults. The compound acts by contact penetrating the insect cuticle and dissolves the cell membranes causing the insect dehydrate and its death (Altinco, 2011).

Management of resistance to prevent or delay the development of resistance to an insecticide and cross resistance to additional insecticides is necessary for increasing the chance of chemical control of *T. absoluta*. Thus, the avoidance of resistance requires the development of pest management programs in which efforts are made to take advantages of natural enemies of pests, plant resistant cultivars, if available, appropriate cultural and physical methods.

Accordingly, diversification of control tactics should be implemented with the minimum use of chemicals. Insecticides should be applied only as needed basis and only used as the last form of control. When insecticides are applied, the way that they are used should be rationalized and optimized to exploit the full diversity of synthetic chemicals and natural products mostly used at rotational basis.

Development of resistance in *T. absoluta* is an important problem in regions where the insect is established. The expanding international trade of plant material not only spread the pest but also spreads the resistance genes associated with the pest (Denholm and Jespersen, 1998). It is possible that the Mediterranean populations of *T. absoluta* already carried gene resistance from South American counterpart populations and thus, may already express high level of resistance to one or multiple insecticide. Indeed, Cifuentes *et al*., (2011), demonstrated high genetic homogeneity of *T. absoluta* populations came from Mediterranean basin and from South America countries using ribosomal and mithochondrial markers.

Our field results (tomato greenhouse) suggest the good performance of the tested compounds (indoxacarb, triflumuron and diafenthiuron). So far, the product indoxacarb tend to be a powerful suppressor of *T. absoluta* larvae.

Indoxacarb is reported by several authors as a powerful insecticide in managing many Lepidopteran pests. Wakil *et al*. (2009) in their study for the management of the pod borer, *Helicoverpa armigera* Hubner (Lepidoptera : Noctuidae) in Pakistan showed the integration of weeding, larvae hand picking and indoxacarb sprays was the most effective in reducing the larval population, pod infestation and maximum grain yield. Also, in Cameroon, Brévault et *al*., (2008) reported a good efficacy of indoxacarb as a larval insecticide of *H. armigera*.

In the United Kingdom, three insecticides were registered for the control of *T. absoluta* under protected tomato, pepper and aubergine: *Bacillus thuringiensis var. kurstaki*, indoxacarb and spinosad (FERA, 2009).

Indoxacarb belongs to a novel class of insecticides, the oxadiazines. It a broad spectrum non-systemic insecticide active especially against Lepidoptera. Indoxacarb affects insect primarily through ingestion but also by contact with treated plant surface. It kills by binding to a site of sodium channels and blocking the flow of sodium ions into nerve cells. The result is impaired nerve function, feeding cessation, paralysis and death (Wing *et al*, 2000).

5. Conclusions

T. absoluta has been a serious pest of tomatoes in Tunisia since the autumn 2008. Farmers have gradually come to understand that conventional insecticides such as organophosphates and carbamates are not effective against the insect. Even though more expensive compared with other insecticides, spinosad (Tracer) is now the widely used bio-insecticide to manage the insect.

It is not the intent in this study to advocate one insecticide over another but to enlarge the array of effective insecticide and bio-insecticides with different modes of action. These studies clearly demonstrated the efficacious of several chemicals such as spinosad, abamectin, emamectin benzoate, triflumuron and diafenthiuron. Although, plant extracts such as Armorex and Deffort show mild efficacy in controlling *T. ab*soluta larvae, they can be used in conjunction with chemical products and integrated in a whole program of control.

The efficacies of sprayings using mixtures of natural products and synthetic chemicals for the control of the pest are planned in our laboratory studies. Indeed, insecticides that work in synergy when mixed together are an avenue to explore in *T. absoluta* control. It has been proposed that pesticides mixtures with different modes of action may delay the onset of resistance developing in pest populations (Bielza *et al.*, 2009). However, some problems need to be considered when two or more insecticides are mixed together especially phyto-toxicity.

The use of insecticides to control *T. absoluta* must not divert attention from the implementation of alternative pest management strategies including cultural, mass-trapping and biological control that can reduce reliance to chemical products.

Chemical pesticides continue to be an important component of insect pest management even with the development of other control methods (mass-trapping, plant resistance…). The use of insecticides based on different chemistries and with varying modes of action is an important component of an integrated pest management strategy. Hence, insecticides will continue to be an integral component of pest management programs due mainly to their effectiveness and simple use. However, the principal factor account for the possible reluctance to shift to the newer insecticides is the high cost.

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7. References

Abbott W.S. 1925. A method for computing the effectiveness of an insecticide. J. Econ. Entomol. 18:265-267.

Aliferis K.A., & Jabaji, S., 2011. Metabolomics- A robust bioanalytical approach for the discovery of the modes of action of pesticides: A review. Pesticide Biochemistry and physiology. 100: 105-117.

Altinco, 2011. www.altinco.org. Accessed on July 7, 2011.

Bielza P., Fernández E., Graválos C., and Albellán J., 2009. Carbamates synergize the toxicity of acrinathrin in resistant western flower thrips (Thysanoptera: Thripidae). J. Econ. Entomol. 102: 393-397.

Bond J.G., Marina C.F., and Williams T., 2004. The naturally derived insecticide Spinosad is highly toxic to *Aedes* and *Anopheles* mosquito larvae. Medical and Veterinary Entomology 18: 50-56.

Brévault B., Oumarou Y., Achaleke J., Vaissayre M. & Nebouche S. 2008. Initial activity and persistance of insecticides for the control of bollworms (Lepidoptera : Noctuidae) in cotton crops. Crop Protection. 28: 401-406.

Campbell C.D., Walgenbach J.F., and Kennedy G.C., 1991. Effect of parasitoids on Lepidopterous pests in insecticide-treated and untreated tomatoes in western North Carolina. J. Econ. Entomol. 84: 1662-1667.

Cifuentes D., Chynoweth R., and Bielza P., 2011. Genetic study of Mediterranean and South American populations of tomato leafminer *Tuta absoluta* (Povolny, 1994) (Lepidoptera: Gelechiidae) using ribosomal and mitochondrial markers. Pest. Manag. Sci. Wileyonlinelibrary.com. DOI10.1002/ps.2166.

Cook D.R. Leonard B.R., and Gore J., 2004. Field and laboratory performance of novel insecticides against armyworms (Lepidoptera: Noctuidae). Florida Entomologist. 87(4): 433-439.

Davidson W.M. 1930. Rotenone as a contact insecticide. J. Econ. Entomol. 23 : 868-874.

Denholm I., and Jepersen J.B ., 1998. Insecticide resistance management in Europe: recent developments and prospects. Pesticide Science. 52: 193-195.

DGPA, Direction Générale de la Production Agricole 2009. Tunisian Ministry of Agriculture. Annual report.

Collier T.R., & Vanstynwyk R., 2003. Olive fruitfly in California: prospects for integrated control. California Agriculture, 57: 28-32.

EstayP. 2000. Polilla del tomate *Tuta absoluta* (Meyrick). http://alerce.inia.cl/docs/informativos/informativo09.pdf. Accessed, 12 May 2010.

Elliott R.H. Benjamin M.C., & Gillott G., 2007. Laboratory studies of the toxicity of spinosad and deltamethrin to *Phyllotreta cruciferae* (Coleoptera : Chrysomelidae). Can. Entomol. 139: 534-544.

El-sheikh E. A. and Aamir M. M., 2011. Comparative effectiveness and field persistence of insect growth regulators on a field strain of the cotton leafworm, *Spodoptera littoralis*, Boisd (Lepidoptera : Noctuidae). Crop Protection. 30 : 645-650.

E-pesticide manual. Version 3.3. 2005. Edited by British Crop Production Council (BCPC). ISBN. 1901396401.

FAO. 2002. Production year book 2000. Vol. 54. FAO. Italy.

FERA, 2009. The food and environment Research Agency. Plant Pest notice. South American tomato moth *Tuta absoluta*. N° 56: 1-4.

Hertlein M.B, Thompson G.D., Subramanyam B. & Athanassiou C.G. 2011. Spinosad : A new natural product for stored grain protection. Journal of stored products research. Doi : 10.1016/j.jspr.2011.01.004 : 1-15.

Ishaaya I., 2010. Biochemical sites of insecticide action and resistance. Isaac Ishaaya (Ed.) Springer-Verlag. Berlin, Heidelberg.

Ishaaya I., Barazani A., Kontsedalov S., & Horowitz A.R., 2007. Insecticides with novel modes of action : mechanism, selectivity and cross resistance. Entomological Research. 37 (Suppl.1) A2-A10.

Jansson R.K., Brown R., Cartwright B. Cox D., Dunbar D.B., Dybas R.A., Eckel C., Lasota J.A., Mookerjee P.K., Norton J.A., Peterson R.F., Starner V.R., & White S., 1996. Emamectin benzoate : a novel avermectin derivate for control of lepidopterous pests. Proceedings : the management of Diamonback Moths and other crucifers pests. Chemical control 171-177. Entomology. Cornell University.

Jansson R.K, Peterson R.F., Mookerjee P.K., Halliday W.R., Argentine J.A., & Dybas R.A., 1997. Development of a novel soluble granule formulation of emamectin benzoate for control of Lepidoptera pests. Florida Entomologist. 80(4): 425-443.

King J.R & Hennesey M.K.1996. Spinosad bait for the Caribbinean fruitfly (Diptera: Tephritidae). Florida Entomologist. 79(4):526- 531.

Labos E., Ochoa J., and Soulier C., 2002. *Cactoblastis cactorum* Berg (Lepidoptera: Pyralidae) preliminary studies for chemical control. Proc. 4th International Conference on Cactus pear and Cochineal. ISHS Acta Horticulturae. Eds. Nefzaoui & Inglese. N° 581: 247-254.

Lietti M.M., Botto E., & Alzogaray R.A., 2005. Insecticide Resistance in Argentine Populations of *Tuta absoluta* (Lepidoptera: Gelechiidae). Neotropical Entomology. 34(1): 113-119.

Maraus P.F., Catapan V., Faria D.S., Filho J.U.T.B., Santos H.S., & Hora R.C. 2008. Eficiência de insecticidas no controle da traça (*Tuta absoluta*) na cultura do tomateiro. Horticultura Brasileira 26: 2929-2933.

(McMillian W.W., Bowman M.C., Starks K.J., & Wiseman B.R., 1969. Extract of Chinaberry leaf as a feeding deterrent and growth retardant for larvae of the corn earworm and fall armyworm. J. Econ. Ent. 62: 708-710.

Meisner J., Ascher K.R.S., Aly R., & Warhben J.D.J. 1981. Response of *Spopdoptera littoralis* (Boisd.) and *Earias insulata* (Boisd.) larvae to azadirachtin and salannin. Phytoparasitica 9:27-32.

Méndez W.A., Valle J., Ibarra J.E., Cisneros J., Penagos D.I., & Williams T., 2002. Spinosad and nucleopolyhedrovirus mixtures for control of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in maize. Biological control 25:195-206.

Mohammed A.S. 2010. New record for leafminer, *Tuta absoluta* (Lepidoptera: Gelechiidae) infested tomato plantations in Kafer El-Sheikh region. J. Agric. Res. Kafer El-Sheikh. Uni. 36(2): 238-239.

OEPP/EPPO. European and Mediterranean Plant Protection Organization. 2005. *Tuta absoluta*. Bulletin OEPP/ EPPO Bulletin. 35:434-435.

Raffa, K.F., 1987. Influence of host plant on deterrence by azadirachtin of feeding by fall armyworm larvae (Lepidoptera : Noctuidae). J. Econ. Entomol. 80: 384-387.

Roditakis E., Papachristos D., & Roditakis N.E. 2010. Current status of the tomato leafminer *Tuta absoluta* in Greece. OEPP/EPPO Bulletin. 40: 163-166.

Salgado V.L., 1998. Studies on the mode of action of Spinosad: insect symptoms and physiological correlates. Pesticides Biochemistry and Physiology. 60: 91-102.

Seal D.R., 2005. Management of melon thrips, *Thrips palmi* Karny (Thysanoptera: Thripidae) using various chemicals. Proc. Fla. State. Hort. Soc. 118: 119-124.

Simmonds M.S.J., and Blaney W.M., 1984. Some neurophysiological effects of azadirachtin on lepidopterous larvae and their feeding response. Natural pesticides from the neem tree (*Azadirachta indica* A. Juss) and other tropical plants. Proc. 2nd. Int. Neem. Conf. Edited by Schmutter H. and Asher K.R.S. German Agency for technical cooperation. Germany.

Sparks T.C., Thompson G.D., Kirst H.A., Hertlein M.B., Larson L.L. Worden T.N & Thibault M.B 1998. Biological activity of Spinosyns, new fermentain derived insect control agents on tobacco budworm (Lepidoptera: Noctuidae) larvae. J. Econ. Ent. 91: 1277-1283.

Siqueira H.A.A, Alvaro A., Guedes R.N.C, & Picanço M.C., 2000a. Insecticide resistance in populations of *Tuta absoluta* (Lepidoptera : Gelechiidae). Agric. Forest. Entomol. 2:147-153.

Siqueira H.A.A, Guedes R.N., & Picanço M.C., 2000b. Cartap resistance and synergism in populations of *Tuta absoluta* (Lepidoptera: Gelechiidae). J. Appl. Ent. 124: 233-238.

Stanley J., Chandrasekaran & Regupathy A. 2005. Basesline toxicity of emamectin and spinosad to *Helicoverpa armigera* (Lepidoptera : Noctuidae) for resistance monitoring. Entomological Research. 39: 321-325.

Strong L., & Brown T.A., 1987. Avermectins in insect control and biology. Bull. Entomol. Res. 77: 357-389.

Takahashi H., Takakusa N., Suzuki J., & Kishimoto T., 1998. Development of a new insecticide, acetamiprid. J. Pestic. Sci. 23: 193-198.

Tomatonews, 2011. http://www.tomatonews.com/resources.html. Accessed March 10, 2011.

Turner N. 1932. Notes on rotenone as an insecticide. J. Econ. Entomol. 25: 1228-1237.

Tuta absoluta. 2011. www.tutaabsoluta.com/agrinewsfull.php?news=3. Accessed 15 March 2011.

Urbaneja A., Vercher R., Navarro V., Garcia-Mari F., & Porcuna J.L., 2007. La polilla del tomate *Tuta absoluta*. Phytoma España. 194:16-23.

Wang D., Cong P.Y., Li M., Qui X.H., & Wang K.Y. 2009. Sublethal effects of Spinosad on survival, growth and reproduction of *Helicoverpa armigera* (Lepidoptera : Noctuidae). Pest. Man. Sci. 65: 223-227.

Wakil W., Ashfaq M., & Ghazanfar M.U., 2009. Integrated management of in chickpea in rainfed areas of Punjab, Pakistan. Phytoparasitica. 37: 415-420.

Walgenbach J.F., Leidy R.B., & Sheets T.J., 1991. Persistence of insecticides on tomato foliage and implications for control of tomato fruitworm (Lepidoptera : Noctuidae). J. Econ. Entomol. 84: 978-986.

Wing K.D., Sacher M., Kagaya Y., Tsurubuchi Y., Muldirig L.,& Connair M., 2000. Bioactivation and mode of action of the oxidiazine indoxacarb in insects. Crop Protection 19: 537-545.

Zapata N., Budia F., Viñuela E., & Pilar M. 2006. Laboratory evaluation of natural pyrehrins, pymetrozine and triflumuron as alternatives to control *Ceratitis capitata* adults. Phytoparasitica 34(4): 420-427.

16

Management Strategies for Western Flower Thrips and the Role of Insecticides[1]

Stuart R. Reitz[1] and Joe Funderburk[2]
[1]*United States Department of Agriculture, Agricultural Research Service, Center for Medical, Agricultural and Veterinary Entomology, Tallahassee, FL,*
[2]*North Florida Research and Education Center, University of Florida, Quincy, FL, USA*

1. Introduction

Today, the western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) is one of the most significant agricultural pests globally because of the damage it is able to inflict on a wide range of crops. Adults and larvae feed by piercing plant tissues with their needle-shaped mandible and draining the contents of punctured cells (reviewed in Kirk, 1997b). Feeding by adults and larvae produces scarring on foliage, flowers and fruits, which results in aesthetic crop damage and disrupts plant growth and physiology. Also, oviposition can produce a wound response in fruiting structures, which reduces the marketability of certain horticultural produce (Childers, 1997). Most importantly, western flower thrips is able to transmit several species of destructive plant viruses in the genus *Tospovirus* (Bunyaviridae). It is the most important vector of *Tomato spotted wilt virus* and *Impatiens necrotic spot virus* worldwide, and it is also known to vector Chrysanthemum stem necrosis virus, *Groundnut ringspot virus* and *Tomato chlorotic spot virus* (Pappu et al., 2009; Webster et al., 2011).

The actual amounts of economic losses attributable to any pest are difficult to determine, but Goldbach and Peters (1994) estimated that *Tomato spotted wilt virus* alone caused over US$1 billion in losses annually on a global basis. This estimate did not include the direct damage caused by western flower thrips, and it still would further underestimate present day losses, as the western flower thrips has continued to spread throughout the world (Kirk & Terry, 2003; Reitz et al., 2011). The state of Georgia, USA compiles estimates of the economic costs of pests to its crops. These estimates include both crop losses and the costs of control measures. From 2001 – 2006, costs from thrips and TSWV for tomatoes (*Solanum lycopersicum*) and peppers (*Capsicum annuum*) have averaged over 12% of the harvested value of those crops per year (Sparks, 2003, 2004, 2005, 2006; Sparks & Riley, 2001, 2002). Over 60% of the total for economic losses from pests and control costs in tomato and pepper are from thrips and tomato spotted wilt virus (Sparks, 2003). In addition, losses in Georgia

[1] The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the United States Department of Agriculture or the Agricultural Research Service of any product or service to the exclusion of others that may be suitable.

in 2006 to ornamentals caused primarily by western flower thrips damage exceeded US$ 15 million. Clearly, these economic assessments show that the western flower thrips is one of the most destructive agricultural pests globally, but its emergence as a major pest has only occurred relatively recently.

The species "*Frankliniella occidentalis*" was first described as a member of the genus *Euthrips* by Theodore Pergande in 1895 from specimens collected in California, where its widespread distribution and abundance across many flowering plants was noted (Pergande, 1895). Subsequently, the species was described under several other names, which have since been synonymized (Mound, 2011). During the early to mid 20th century, the western flower thrips was regularly mentioned as a member of complexes of pestiferous thrips in western North America, but it was generally regarded as a less significant problem than species such as *Taeniothrips inconsequens* (Uzel), *Thrips tabaci* Lindeman, *Scirtothrips citri* (Moulton) and *Heliothrips haemorrhoidalis* (Bouché) (Moulton, 1931). For most of the early 20th century, western flower thrips remained a localized problem in California and other areas of the western USA and Canada, with sporadic problems reported from southern Texas to British Columbia.

One of the first cases of damage attributed to western flower thrips occurred on potatoes grown in the San Gabriel Valley of southern California. Feeding damage from large populations of the western flower thrips was implicated as the cause of curling of new foliage on potato *(Solanum tuberosum)*. The condition, termed potato curly leaf, would lead to significant reductions in tuber size and yield (Crawford, 1915). The recommended control for these thrips infestations was early season applications of Bordeaux mixture combined with extracts of tobacco, which was able to reduce losses when timed appropriately.

Also early in the 20th century, the western flower thrips was recorded as a pest of other vegetable, fruit and nut crops. Riherd (1942) observed oviposition damage on peas grown in the Rio Grande Valley of Texas, which reduced the aesthetic quality of fresh market peas (*Pisum sativum*). Similar spotting patterns, termed "pansy spots", on apples (*Malus domestica*) grown in the northwestern USA were attributed to western flower thrips oviposition (Childs, 1927; Moulton, 1931), as was damage to table grapes (*Vitis vinifera*) grown in California (Jensen, 1973; Yokoyama, 1977). Another significant type of damage to fruit crops resulted from feeding on floral tissues that would cause flower loss before fruit set. Occasionally, larval feeding was known to produce scarring of citrus fruit (Essig, 1926; Woglum & Lewis, 1935). During this period, western flower thrips also was implicated as one of thrips causing damage to alfalfa flowers (*Medicago sativa*), which reduced set seed (Borden, 1915; Seamaxs, 1923).

Later, during the 1950's the western flower thrips began to receive more attention as a pest of seedling cotton (*Gossypium hirsutum*) in the southwestern USA. Extensive use was made of all major classes of insecticides, including organochlorines, organophosphates, and carbamates, during the 1950's – 1960's, to combat this early season threat. Recommendations were made to growers to make multiple applications of insecticides to prevent loss of seedlings from thrips feeding damage. However, control failures with organochlorine insecticides were observed as early as 1960 (Race, 1961). The control failures with organochlorines then led growers to make preventative applications of organophosphates to protect seedlings. These were often made as systemic treatments at planting. However, Race (1965) recognized a significant disadvantage with this approach of intense preventative insecticide use, namely that growers could be making unnecessary applications when the

thrips populations did not warrant treatment. Further complicating management issues were the findings by Shorey et al. (1962) that carbamates actually led to rapid resurgence of western flower thrips populations after applications in cotton, which were likely related to adverse effects of the insecticides on beneficial insects.

Harding (1961a) observed that western flower thrips was the predominant thrips species infesting onions (*Allium cepa*) grown in southern Texas. It was also observed to be the predominant thrips in California onions (Hale & Shorey, 1965). Multiple applications of insecticides, predominately organophosphates, were found to reduce thrips abundance but not to improve yields significantly (Harding, 1961b).

Western flower thrips were noted as a minor pest of other vegetable crops in California. It was confirmed as a vector of *Tomato spotted wilt virus* in the 1930's (see Sakimura, 1962), but viral epidemics caused by western flower thrips apparently were not common at this time, and most damage was a result direct feeding rather than virus transmission (Sakimura, 1961). Similar to the situation with seedling cotton, western flower thrips feeding on young tomato plants was known to reduce photosynthesis and lead to defoliation and plant death (Shorey & Hall, 1963). Thrips feeding on lettuce (Latuca sativa) produces scarred, corky tissue on leaves. Shorey found that organophosphates would reduce populations for a short time after applications, but that populations would rebound soon after applications (Shorey & Hall, 1962). One of the causes identified for the lack of long term control of western flower thrips by insecticides in open field crops was that crops were subject to repeated ongoing dispersal of adults from outside crop fields (Shorey & Hall, 1963). Although tomato spotted wilt epidemics were uncommon in California at this time, Shorey and Hall (1963) also noted the inability of conventional insecticides to reduce Tomato spotted wilt virus transmission within fields because of the repeated dispersal of viruliferous thrips into fields from external sources.

Notably in major review articles concerning floriculture crops, western flower thrips was seldom discussed as a significant pest before the 1970's (Bryan & Smith, 1956; Naegele & Jefferson, 1964; Price et al., 1961), although the damage it caused to greenhouse-grown cut flowers was well recognized in California by the 1930's (Bohart, 1943). At this time, greenhouse growers relied on methods such as intensive sprays of nicotine sulfate or tartar emetic, and fumigation with nicotine, napthalene or calcium cyanide. Fumigation was found to kill adults but was far less effective against immature stages. Therefore, populations could rebound quickly after a fumigation treatment, thus exacerbating overall pest management concerns for growers.

2. Western flower thrips as an invasive species

It is likely that Naegele and Jefferson (1964) did not discuss western flower thrips because of its limited distribution at the time of their review article on floriculture pests. However, the pest status of western flower thrips began to expand rapidly in the late 1970's when growers in California began to experience more extensive damage to cut flower crops from thrips feeding and virus transmission (Robb, 1989). Because of the exceedingly low damage thresholds for these crops, growers responded to the threat with intensive insecticide treatments, leading to the rapid development of resistance to all major classes of insecticides available at the time, including pyrethroids, carbamates, organophosphates and abamectin (Immaraju et al., 1992).

This insecticide-resistant strain(s) of western flower thrips that originated in California is thought to then have spread around the world in association with the globalization of the cut flower and horticulture industries (Bonarriva, 2003; Huang, 2004). The western flower thrips was established throughout agroecosystems of eastern North America by the mid 1990's, and its spread through Europe was even more rapid (Kirk & Terry, 2003). It was first recorded in the Netherlands in 1983 (Mantel & Van de Vrie, 1988), and large outbreaks were observed in almost all European countries by 1990 (Kirk & Terry, 2003). In northern areas of Europe, western flower thrips is largely restricted to glasshouses[2] because climatic conditions prevent persistent populations from establishing (McDonald et al., 1997). In more southerly areas around the Mediterranean basin, populations have become established in open field crops, which facilitates the repeated colonization of protected crops (Brødsgaard, 1993; Kontsedalov et al., 1998). Since the distribution review by Kirk and Terry (2003), western flower thrips has continued to spread to new regions. Significantly, it is now a widely established pest in China (Reitz et al., 2011). As a reflection of its current cosmopolitan distribution and pest status, western flower thrips, a native of North America, is one of the most frequently intercepted insect species at USA ports of entry (Nickle, 2004).

Recently, the taxonomic status of *Frankliniella occidentalis* has been called into question. Molecular evidence indicates that "*Frankliniella occidentalis*" is a complex of two cryptic, sympatric species (Rugman-Jones et al., 2010). Rugman-Jones et al. (2010) have designated the two species as the lupin (L) and glasshouse (G) species. Neither of these genetic species corresponds to the historic morphological descriptions of the species of *F. moultoni* (Hood) or *F. occidentalis* (Pergande). The "lupin" and "glasshouse" designations are based on the similarity of certain individuals with a "western flower thrips" population that has been associated with *Lupinus arboreus* in New Zealand since the 1930's (Martin & Workman, 1994) and other individuals corresponding to pest strains found in European glasshouses (Brødsgaard, 1994). This recognition of species diversity within "*F. occidentalis*" adds uncertainty as to which species is under consideration in earlier literature. However, circumstantial evidence suggests that recent pest problems are largely the result of the highly invasive "glasshouse" type (Martin & Workman, 1994; Rugman-Jones et al., 2010).

Ironically perhaps, responses to these new, expanding problems from invasive western flower thrips populations were similar to historic control efforts attempted in the western USA; namely, there was a heavy reliance on insecticide use for control. Despite pervasive attempts to control western flower thrips with the widespread, intense use of insecticides, there are many factors that limit the efficacy of insecticides. These include ecological, behavioral and physiological factors, and these need to be appreciated and understood to place insecticide use in a proper context. Ecologically, western flower thrips is highly polyphagous and capable of reproducing on numerous host plants (Northfield et al., 2008; Paini et al., 2007). As large populations can develop on non-crop hosts, mass dispersal into crops occurs, whether open field crops (Pearsall & Myers, 2001; Puche et al., 1995; Ramachandran et al., 2001) or crops in protected environments (Antignus et al., 1996). The potential for continual recolonization of crops limits the observed field efficacy of insecticides (Eger et al., 1998; Reitz et al., 2003). This ongoing dispersal means that even repeated insecticide applications have little utility in reducing pest damage, especially in high value crops with low damage thresholds (Bauske, 1998; Kontsedalov et al., 1998). Once

[2] The terms "greenhouse" and "glasshouse" are used interchangeably in this article for convenience, although they are not necessarily structurally equivalent.

having landed on plants, western flower thrips preferentially reside within flowers or other concealed, protected places on plants (Hansen et al., 2003; Kirk, 1997a). This thigmotactic behavior of thrips limits their exposure to many foliar applied insecticides. Also, the anthophilous nature of western flower thrips limits their exposure to systemic insecticides, which are not readily transported into floral tissues (Cloyd & Sadof, 1998; Daughtrey et al., 1997). Therefore, some of the most effective materials are those with translaminar properties, which increase the probability of thrips concealed in flowers actually ingesting toxins (Kay & Herron, 2010).

3. Insecticide use and insecticide resistance

While delivering toxins to western flower thrips can be problematic, and the species behavior and ecology can minimize exposure to insecticides, the species is well suited to evolve resistance to multiple classes of insecticides. Since the first reported case of control failures with insecticides (toxaphene, an organochlorine, Race, 1961), there have been numerous incidences of resistance reported to all major classes of insecticides from all regions of the world (Bielza et al., 2007b; Brødsgaard, 1994; Dağli & Tunç, 2007; Immaraju et al., 1992; Jensen, 2000a; Kay & Herron, 2010; Morishita, 2001; Robb et al., 1995; Weiss et al., 2009; Zhao et al., 1995). Resistance has not only developed against insecticides targeting western flower thrips, but also insecticides used to treat other pest species. Correspondingly, a number of different resistance mechanisms have been characterized to date, including metabolic detoxification, reduced penetration, altered target site resistance, and knockdown resistance (Bielza, 2008).

One class of insecticides where resistance problems have been particularly acute is the pyrethroids. Robb (1989) recorded control failures with pyrethroids in ornamental greenhouses in California. Management became so difficult, with a lack of alternative insecticides that growers returned to using legacy non-synthetic insecticides, such as nicotine sulfate. Despite these early reports regarding difficulties with pyrethroids, they have continued to be used against western flower thrips extensively, with the same outcome of resistance development (Broadbent & Pree, 1997; Espinosa et al., 2002b; Frantz & Mellinger, 2009; Immaraju et al., 1992; Seaton et al., 1997; Thalavaisundaram et al., 2008; Zhao et al., 1995). In these cases, the development of resistance to pyrethroids has tended to occur rapidly. Resistance to pyrethroids led Australia to abandon their use for western flower thrips management less than eight years after the pest was first detected (Herron & Gullick, 2001). Likewise, pyrethroids are no longer recommended for use in Turkish greenhouses because of the rapid development of resistance and cross resistance to other chemicals (Dağlı & Tunç, 2008).

Resistance to pyrethroids is primarily derived from metabolic detoxification pathways (Broadbent & Pree, 1997; Espinosa et al., 2005; Maymó et al., 2006; Zhao et al., 1995). A broad range of enzymatic detoxification pathways to detoxify pyrethroids have been identified, including cytochrome P450 monooxygenases, glutathione S-transferases and esterases. The predisposition of western flower thrips to evolve resistance based on metabolic detoxification is likely a product of its polyphagous nature. Because individuals move from host to host, they are likely to encounter a variety of plant defensive chemicals. Therefore, it is adaptive to have multiple means to contend with the unpredictable suite of host plant defenses that they may encounter (Rosenheim et al., 1996).

These inherent metabolic detoxification pathways predispose the western flower thrips to overcome pyrethroids and other classes of insecticides. Adding to the factors that make western flower thrips amenable to developing insecticide resistance are its rapid development rate so that populations may pass through several generations within a single cropping system, its high fecundity, so that resistant females can produce many offspring, and the haplodiploid sex determination characteristic of thrips (Reitz, 2009). In this type of sex determination, females are diploid, but males are haploid. Because males are haploid, their alleles are exposed directly to selection, which enable alleles for resistance to become fixed rapidly in a population (Denholm et al., 1998).

Insecticide resistance in western flower thrips is a complex phenomenon. It is important to note that resistance to a particular insecticide may not derive from a single trait and can involve multiple pathways (Jensen, 1998). For example, resistance to diazinon, an organophosphate, and methiocarb, a carbamate, has been linked to both metabolic detoxification and altered target site sensitivity (Jensen, 2000b; Zhao et al., 1994). In addition, distinct populations can evolve resistance to a particular insecticide through different mechanisms (Jensen, 2000b; Thalavaisundaram et al., 2008). However, a single metabolic mechanism may confer cross resistance between different classes of insecticides. Espinosa et al. (2002a) found that single metabolic pathway appears to confer resistance to pyrethroids and carbamates. In turn, Bielza et al. (2007a) proposed that such a single detoxification mechanism for the pyrethroid acrinathrin and for carbamates could be exploited, by using carbamates as synergists to increase the activity of acrinathrin through competitive substrate inhibition. These variable pathways complicate the prediction of resistance development and the potential for cross resistance to multiple classes of insecticides in any population.

Individuals carrying resistance alleles are often considered to be at a fitness disadvantage in a population when the particular insecticide to which they are resistant is not used (i.e., in the absence of particular selection pressures) (Georghiou & Taylor, 1986). When such a fitness disadvantage is present in resistant individuals, reversion to a susceptible population should occur soon after removal of the specific insecticide (selective pressure). However, this outcome may not always be the case for western flower thrips. There are cases of long term maintenance of resistance in the absence of insecticide exposure. Kontsedalov et al. (1998) reported that resistance to the pyrethroid cypermethrin did not decline after more than seven years of non-exposure for a laboratory colony. Likewise, resistance to organophosphates can be maintained in field and laboratory populations for several years without exposure (Brødsgaard, 1994; Robb, 1989). Bielza et al. (2008) compared fecundity, fertility, longevity, and egg-to-adult developmental time in populations of western flower thrips from Spain that were resistant and susceptible to acrinathrin (pyrethroid) and spinosad (a spinosyn formulation, see below), and found that resistance to either material did not carry significant fitness costs, at least in terms of the parameters they measured. Therefore, resistance may be expected to develop rapidly and be maintained for long periods of time in the absence of the use of an insecticide, and these scenarios must be taken into account in developing insecticide resistance management programs, which are integral to integrated pest management (IPM) programs.

Given the ongoing issues with management failures and resistance development with synthetic insecticides, and the cancellation of registrations for many other synthetic insecticides as a result of the Food Quality and Protection Act of 1996 (US EPA, 1996), there is a limited pool of efficacious insecticides for use against western flower thrips. Consequently, there is intense interest in developing new, alternative insecticides. Some of

the most efficacious insecticides against western flower thrips in recent years have been spinosyns, which are metabolites derived from fermentation of the actinomycete bacterium, *Saccharopolyspora spinosa* (Sparks et al., 1999). Spinosyns are in a group of insecticides with a novel mode of action, the nicotinic acetylcholine receptor (nAChR) allosteric activators (Group 5 – Insecticide Resistance Action Committee) (IRAC International MoA Working Group, 2011). The unique mode of action of spinosyn-based insecticides and their translaminar properties have made them highly effective against western flower thrips. The first products with spinosad as the active ingredient (a combination of spinosyns A and D; Dow AgroSciences, Indianapolis, IN) were registered for use in the late 1990's (Thompson et al., 2000). This was followed later by the release of spinetoram in 2008. Given the effectiveness of spinosyns and the lack of effective alternatives, growers tended to place an overreliance on spinosad, making them a victim of their own success. The first evidence of resistance to spinosyns was detected in western flower thrips populations in Australia by 2002 (Herron & James, 2005), Spain by 2003 (Bielza et al., 2007b) and the US by 2006 (Weiss et al., 2009). Spinosyn resistance in western flower thrips appears to be based on altered target site resistance, with spinosad resistance in a Spanish population based a single locus, autosomal recessive trait (Bielza et al., 2007c). The evidence that spinosad resistance is a recessive trait means that this resistance may not be stable, which would facilitate reversion to susceptibility in populations (Weiss et al., 2009). However, as with other resistance cases, mechanisms and genetics of spinosyn resistance likely vary across populations. Zhang et al. (2008) reported that spinosad resistance in a population from Japan is likely polygenic. Perhaps, more troubling is the potential cross resistance to other insecticides that Zhang et al (2008) found in their spinosad resistant strain. This potential will necessitate further caution in managing the use of spinosyn products.

In efforts to broaden the range of insecticides available for western flower thrips management, there has been interest in adapting other existing chemistries not previously labeled for use against western flower thrips. Neonicotinoids, which have been used since the 1990's to manage various types of other sucking insect pests, have recently received attention for a potential role in western flower thrips management. In experimental trials in Australia, Broughton and Herron (2009) found that two neonicotinoids, acetamiprid and thiamethoxam, were as effective as spinosad against larvae and adults of western flower thrips in pepper and lettuce. However, none of the tested insecticides, including spinosad, were effective in reducing the abundance of western flower thrips adults in tomato. Coutts and Jones (2005) found that drenching lettuce seedlings with neonicotinoids, in particular imidacloprid, just before transplanting reduced the incidence of tomato spotted wilt significantly in field trials. However, during their field trial, the predominant vector species were *Frankliniella schultzei* (Trybom) and *Thrips tabaci,* with very few western flower thrips found. Interestingly, their results showed that there were minimal effects of the neonicotinoids on the abundance of any of the thrips species. Likewise, western flower thrips populations showed little effect from applications of another neonicotinoid, dinotefuran, in pepper and strawberry (Dripps et al., 2010). It is possible that in these cases, mortality induced by the insecticide was counteracted by the loss of natural enemies of thrips from the insecticide applications. Alternatively, disease reductions observed by Coutts and Jones (2005) may have resulted from sublethal effects of the insecticides on thrips. Imidacloprid was found to actually enhance feeding of western flower thrips on tomato foliage while it reduced feeding by another TSWV vector, *Frankliniella fusca* (Hinds) (Joost & Riley, 2005). Likewise, the anthranilic diamide, cyantraniliprole, which disrupts

insect feeding activity, reduced TSWV transmission by *F. fusca,* but had no effect on transmission by western flower thrips (Jacobson & Kennedy, 2011). Therefore, more thorough evaluations should be made of the use of neonicotinoids specifically for western flower thrips management in different cropping systems. Given these results, it is important also not to extrapolate from the effects of insecticides on one thrips species, even congeneric ones, to *F. occidentalis.*

Despite indications of resistance to abamectin developing (Immaraju et al., 1992), it and related materials in the avermectin class have continued to receive interest as a means to manage western flower thrips in greenhouse and open field crops. Spiers et al. (2006) recently found that abamectin was as effective as spinosad in reducing western flower thrips feeding damage to gerbera daisies (*Gerbera jamesonii*). Overall flower quality was high not only because of the low amount of damage but also because of the lack of phytotoxic effects. The abamectin derivative, emamectin benzoate, has also been found to have efficacy against western flower thrips (Ishaaya et al., 2002). This material was found to be more potent than the parent material abamectin, and it was more effective against larvae than against adults. An advantageous property of abamectin and emamectin benzoate is that they are translaminar, increasing the likelihood that toxins will reach concealed thrips adults and larvae. However, other results have shown that these avermectins are not effective against western flower thrips in field trials (Kay & Herron, 2010). They found that their test population of western flower thrips was highly susceptible to abamectin and emamectin benzoate in direct exposure assays so that the field failures could result from difficulties in applying adequate doses of avermectins to plants in the field (Broughton & Herron, 2007).

Other newly developed insecticides with novel modes of action continue to be evaluated for their potential role in western flower thrips management. Pyridalyl, which has not been classified as yet for its mode of action, is more toxic to larvae than to adults of the western flower thrips, but it is compatible with biological control agents such the predatory bug *Orius strigicollis* (Poppius) (Isayama et al., 2005). This feature makes it an attractive insecticide for rotational use in an overall integrated pest management program. Pyridalyl is registered for greenhouse ornamentals in the USA and certain other crops elsewhere in the world. Likewise, the pyrrrol chlorfenapyr is registered for greenhouse ornamental and vegetables in the USA, but is approved for use in other situations globally. It has shown efficacy comparable to spinosad in greenhouse trials conducted in Australia (Broughton & Herron, 2009). Both pyridalyl and chlorfenapyr have translaminar properties. The systemic insecticide fipronil, a phenylpyrazole, is effective against western flower thrips larvae, and to a lesser extent, adults (Kay & Herron, 2010). In an interesting approach to synergizing insecticides, Cook et al. (2002) found that the addition of dodecyl acetate, a component of the western flower thrips alarm pheromone, increased the efficacy of fipronil in field trials against western flower thrips in strawberry (*Fragaria* × *ananassa*).

There has also been recent interest in the use botanically derived insecticides for use against the western flower thrips. Certain essential oils can help reduce the incidence of tomato spotted wilt in tomato (Reitz et al., 2008), and other *Chenopodium* based materials have shown efficacy under greenhouse conditions (Chiasson et al., 2004). *Chenopodium* based products, while not highly toxic under open field conditions, provide sufficient suppression of western flower thrips larvae to warrant inclusion in insecticide rotation schemes (Funderburk, 2009). These essential oil products tend to have little negative impact on natural enemies, so they may be compatible in overall IPM programs (Bostanian et al., 2005). One drawback in the use of plant essential oils has been that concentrations of oils needed to

have lethal insecticidal properties to pests can be phytotoxic (Cloyd et al., 2009, S. R. Reitz, unpublished).

There has been considerable interest in the use of microbial insecticides against western flower thrips (Butt & Brownbridge, 1997). Although experimental work has demonstrated the effectiveness of these materials and natural epizootics have been recorded (Vacante et al., 1994), there has been limited commercial success with them. Several products are available for commercial use (Shah & Goettel, 1999), and new formulations are still being developed (e.g., Zhang et al., 2009).

There has been a long history of insecticide use against the western flower thrips. Yet, there has only been a limited number of efficacious available at any given time, and none have been able to serve as a stand-alone management tactic. Although several new insecticides have been reported to have efficacy against western flower thrips, there is still a limited suite of insecticides that are effective. This limitation is likely to be an ongoing constraint because of the cost of developing and registering new insecticides. Therefore, to maintain the utility of efficacious insecticides as a part of IPM programs for western flower thrips, it is critical to take conservative and judicious approaches to the use of these insecticides.

4. Insecticide resistance management

Given the history of insecticide use against western flower thrips, resistance development is more than likely to occur to any insecticide, regardless of mode of action. Therefore, it is critical to develop strategies to employ them effectively. A key element in this regard is proper insecticide resistance management programs to maintain efficacy for as long as possible. In general, insecticide resistance management programs for western flower thrips do not differ conceptually from those designed for other pests. The basic concept is to rotate among insecticides with different modes of action at appropriate intervals to delay or inhibit the evolution of resistance within a pest population.

Most current insecticide resistance management plans recommend rotation of chemical classes after every generation of thrips (Broadbent & Pree, 1997; Herron & Cook, 2002; Robb et al., 1995). In Australia, the initial recommendations for insecticide resistance management were for growers to alternate among insecticides from different chemical classes with each application, a practice recommended in other regions (Funderburk, 2009). However, Herron and Cook (2002) proposed that this simple strategy would not be effective because of the long term persistence of resistance in populations to cypermethrin, a pyrethroid. They argued that reversion to a susceptible population would not occur before an insecticide was used again, rendering that material ineffective. It is also possible that alternating chemical classes too frequently (within a generation) could more readily select for individuals with resistance to multiple insecticides. In a subsequent study, Broughton and Herron (2007) advocated a three-consecutive spray program of a particular insecticide before rotating to a different chemical class. A key component to this strategy was that the three-consecutive applications needed to be made within a single thrips generation to gain the maximum effectiveness for that treatment.

The actual implementation of such an approach is limited by the continuous, overlapping generations present within a crop (Reitz, 2009), and may best be interpreted as rotating chemistries on an appropriate time interval (approximately 3 weeks). Bielza (2008) further cautioned that it is not simply enough to rotate among different chemistries. Rather rotation schedules should be based on known resistance mechanisms to avoid problems with cross

resistance. An example would be to rotate from chemistries in which metabolic resistance is likely to develop to chemistries in which target site resistance is likely to develop. The more types of resistance modes that can be built into a rotation plan, the more effective each material would be expected to be. Unfortunately in some cropping systems, growers may be faced with having only one or two efficacious classes of insecticides, which increases the risk of resistance development (Broughton & Herron, 2007). Many growers will make applications that are mixtures of more than one insecticides (Cloyd, 2009a). This is done either to combat more than one pest at a particular time, or in the belief that better control of a particular pest can be achieved with mixtures. However, Bielza (2008) also cautioned against using mixtures of insecticides because the structure of western flower thrips populations and resistance mechanisms may actually increase rates of resistance development when mixtures are used.

Bielza (2008) outlined a general resistance management protocol that also serves as a foundation for a sound IPM program. The four recommendations are to: 1) apply insecticides only when required; 2) make accurate and precise insecticide applications; 3) diversify the types of management methods that are used in a crop; and 4) conserve natural enemies. In addition, resistance monitoring needs to be conducted on an ongoing basis so that insecticides can be quickly removed from use before complete failures occur, and so that susceptibility to those materials can be restored. The proper stewardship of insecticide use will help to forestall the development of resistance in western flower thrips populations, as well as populations of other pest species inhabiting particular crops. Even with sound insecticide resistance management programs in effect, it is clear that insecticides cannot function as a stand-alone control method for western flower thrips, and most authors have advocated that insecticides not be used as a stand-alone management tactic. In fact, there cannot be a reliance on any single tactic, and truly integrated management approaches need to be employed.

5. Western flower thrips IPM in open field vegetables in the Southeastern USA

IPM programs developed in Florida for open field vegetable crops are an example of the evolution management programs for the western flower thrips and tomato spotted wilt virus. The development of these IPM programs has relied on a thorough understanding of western flower thrips biology and ecology. Perhaps, the most important aspect for successful management is the recognition that complete control of western flower thrips and elimination of damage is not attainable. Rather, the goal should be to manage thrips within acceptable limits that do not result in economically significant damage, and this goal has become the focus of current western flower thrips management programs (Funderburk, 2009).

Northern Florida, and the rest of the southeastern USA, is a major producer of fresh market tomatoes and peppers, although farms in this region tend to be relatively small (10 – 100 ha) and dispersed throughout the landscape (Bauske, 1998). Vegetable crops in the region are grown on beds covered with plastic mulches (Castro et al., 1993). Crops are started from transplants, with the typical crop growing in the field for 12 – 14 weeks. Tomato and pepper crops in the region did not experience pest problems from thrips until the western flower thrips invaded in the 1980's (Beshear, 1983; Olson & Funderburk, 1986). Initially, damage from western flower thrips was observed from oviposition and direct feeding of adults and

larvae on developing tomato fruits, which reduce their aesthetic quality and marketability (Ghidiu et al., 2006; Salguero-Navas et al., 1991). Similar scarring damage from feeding can occur on pepper fruit (Funderburk et al., 2009). However, soon after the invasion of the western flower thrips, epidemics of tomato spotted wilt began to occur throughout the southeast (Csinos et al., 2009) and crops remain at risk if proper management is not employed (Reitz et al., 2008). It is important to note that the most prevalent *Frankliniella* species in Florida and the southeastern USA are *F. tritici* (Fitch) and *F. bispinosa* (Morgan) (in southern Florida, Hansen et al., 2003), but these species do not cause the damage that western flower thrips do in vegetable crops.

The extensive crop losses caused by western flower thrips and *Tomato spotted wilt virus* have spurred considerable research to develop effective management programs. Understandably though, when western flower thrips and tomato spotted wilt first emerged as problems, tomato and pepper growers in the region responded with intensive insecticide treatments in attempts to prevent disease spread. By the 1990's growers in northern Florida were making an average of 16 separate applications of insecticides, with each application often being a mixture of multiple insecticides (Bauske, 1998). Despite such intense insecticide treatments, attempts at vector control through insecticides did not substantially reduce the problems. This lack of success results from the fact that most virus transmission in these crops is a result of primary spread of the pathogen – that is, infection comes from viruliferous individuals that disperse into the crop from external sources (Gitaitis et al., 1998; Puche et al., 1995). Furthermore, *Tomato spotted wilt virus* transmission occurs in as little as 5 minutes of an adult thrips feeding on a plant (Wijkamp et al., 1996). The following is a description of key pest management tactics that have been developed to successfully manage thrips and *Tomato spotted wilt virus* in open field fruiting vegetables.

5.1 Scouting

Because thrips species vary in their pest status, and because insecticides can differentially impact populations of native flower thrips and the invasive western flower thrips, it is necessary to accurately identify the species in order to make and evaluate management decisions. Thrips can be easily sampled by collecting flower samples into containers with alcohol. These samples can then be examined under a microscope with at least 40X magnification to determine the species. Various identification keys are available to assist with species identifications at this level (e.g., Frantz & Fasulo, n.d.). Periodic sampling can be used to assess shifts in the relative abundance of species of thrips throughout the growing season. When done systematically, this sampling is invaluable for determining the need for any insecticide application and the effects of such applications.

Sampling for crop management scouting purposes can be accomplished by counting the thrips from samples of ten flowers collected from each of several locations throughout a field. The number of samples needed to collect depends, in part, on field size. Because of the potential for scarring damage to fruit, it is also important to examine small, medium, and large fruits for thrips, with care taken to look under the calyx because of the thigmotactic behavior of thrips. Small fruits especially need to be inspected frequently as the eggs generally are laid during the flower stage, and larvae on the small fruit are the first indication of a developing problem. Again, it is important to sample fruit from several locations in each field.

5.2 Economic thresholds

Economic thresholds have been developed for thrips management in fruiting vegetable crops, including tomato, pepper and eggplant (Funderburk, 2009). These thresholds primarily apply to oviposition damage by female western flower thrips and to the feeding damage caused by adult western flower thrips and larvae of this and other thrips. Consequently, species identification in scouting is critical for the use of thresholds. Thresholds can guide growers for making therapeutic insecticide applications to mitigate these types of damage. However, growers must be aware that therapeutic treatments can do little to mitigate virus transmission. Therefore, preventative tactics must be employed to manage the primary spread of disease in crop fields. Secondary spread may be managed by scouting for larvae and treating, as appropriate.

Tomato: Although adults of all *Frankliniella* species that occur in Florida feed on pollen, petals and other floral structures in tomato, this feeding injury does not result in economic damage. However, once feeding by adults of western flower thrips and larvae of all species commences on immature fruits, it can produce "flecking" damage, which becomes apparent as the tomatoes ripen (Ghidiu et al., 2006). Oviposition in developing tomato fruit from western flower thrips also causes aesthetic damage (Salguero-Navas et al., 1991). Whereas 25 adults of native thrips (*F. tritici* and/or *F. bispinosa*) per bloom do not cause damage, one western flower thrips adult per flower is the threshold at which growers need to take action. An average of up to two larvae per small, medium or large fruit can be tolerated, but growers should take action at these thresholds (Funderburk, 2009; Funderburk et al., 2011).

Pepper and eggplant: As with tomato, adults of *F. tritici* and *F. bispinosa* cause little, if any, damage to pepper and eggplant, even with densities of 25 per bloom, and they beneficially outcompete western flower thrips and melon thrips, *Thrips palmi* Karny (Funderburk, 2009). Direct feeding damage from adults of western flower thrips and melon thrips is less severe than in tomato, and oviposition in immature pepper and eggplant fruit by western flower thrips does not cause the damage that is typical in tomato and some other crops. Therefore, higher thresholds can be tolerated in pepper and eggplant than in tomato. Up to six (6) western flower thrips and/or melon thrips adults per flower can be tolerated without damage. Once fruits begin to develop, growers need to be aware of scarring damage that adults of the western flower thrips and the melon thrips and larvae of all species may cause. Up to two larvae per small, medium, or large fruit on average in a field are tolerable. Growers should be prepared to take action if larval populations exceed two per fruit (Funderburk 2009). Because of the critical role that *Orius* spp. play in suppressing thrips populations and secondary virus spread, their populations should be monitored in scouting programs, and considered when assessing the need for insecticide treatments.

5.3 Biological control

Despite certain similarities between crops of tomato and pepper, there are fundamental differences in interactions between thrips and these two plant species. These differences mean that management programs must be designed for each crop. Adult western flower thrips readily colonize pepper and tomato (Baez et al., 2011). Tomato, though, is not a significant reproductive host for western flower thrips (Funderburk, 2009), but pepper can be a good reproductive host for these thrips (van den Meiracker & Ramakers, 1991). Consequently, there is the potential for secondary virus spread from within the crop (Gitaitis et al., 1998). However, Funderburk et al. (2000) demonstrated that the predator

Orius insidiosus (Say) colonizes peppers and can effectively suppress thrips populations in the crop (Funderburk et al., 2000; Ramachandran et al., 2001). In particular, *O. insidiosus* preferentially preys on western flower thrips over the native species *F. tritici* and *F. bispinosa* (Baez et al., 2004; Reitz et al., 2006). Therefore, conservation of these valuable naturally occurring biological control agents can significantly reduce pest problems in pepper and related crops (e.g., eggplant, *Solanum melongena*) and has become a cornerstone of IPM for pepper production (Funderburk et al., 2009). One of the keys to conservation of *Orius* species is to use insecticides that are minimally toxic to *Orius* spp., whether for thrips management or for management of other pests (Reitz et al., 2003). In contrast to pepper, *Orius* species do not have an affinity for tomato (Baez et al., 2011; Pfannenstiel & Yeargan, 1998), and so this naturally occurring biological control is not available for tomato.

5.4 Interspecific competition

Biotic limitations on western flower thrips populations can come from other species of thrips as well as predators, such as *O. insidiosus*. Recent studies have shown that interspecific competition from native thrips limits the larval survivorship of western flower thrips (Paini et al., 2008). In a survey conducted in northern Florida, Northfield et al. (2008) observed that over 75% of thrips collected from a range of uncultivated hosts were the native species *F. tritici,* with only 1% being western flower thrips. Similar results have been observed in crop fields where two-thirds or more of thrips in untreated or spinosad-treated pepper are the native species. Yet, the demographics differ in pyrethroid treated plots, where western flower thrips predominant (Hansen et al., 2003; Reitz et al., 2003). Because the native species *F. tritici* and *F. bispinosa* do not cause the economic damage that western flower thrips do, and because they outcompete western flower thrips, their conservation contributes to overall pest management. This difference in pest status among the species is why species identifications are an essential component of scouting in IPM programs.

5.5 Host plant location and ultraviolet reflective mulches

Thrips locate host plants primarily through a combination of visual cues, with anthophilous thrips tending to be attracted to colors of flowers. Western flower thrips are attuned to spectral radiation in the ultraviolet range (~365 nm) and in the yellow-green range (~540 nm) (Matteson et al., 1992). The yellow-green sensitivity is thought to play a role in long distance orientation to plants, and the ultraviolet sensitivity is part of the visual system to distinguish flowers. Anthophilous thrips, such as western flower thrips, are attracted to colors of flowers, especially white, blue and yellow flowers with low ultraviolet reflectance (Antignus, 2000). Therefore, increasing the reflectivity in ultraviolet range of the spectrum can repel thrips.

The ultraviolet reflective mulches available for the raised-bed plastic mulch production system of Florida are effective in repelling migrating adults of the western flower thrips, and this repellency reduces the primary and secondary spread of tomato spotted wilt. The use of ultraviolet reflective mulch also reduces the influx of the native thrips, *F. tritici* and *F. bispinosa,* but not disproportionately to reductions in western flower thrips (Momol et al., 2004; Reitz et al., 2003). Ultraviolet reflective mulches are most effective early in the crop season before the plant canopy begins to cover the mulch and reduce the surface area available for reflectance. Application of certain bactericides/fungicides and other pesticides also reduces the ultraviolet reflectance and hence the efficacy of the mulch. A single application of copper and mancozeb

for bacterial or fungal control can reduce the reflectance by nearly 50%. Repeated applications can consequently lead to higher incidences of tomato spotted wilt (S. R. Reitz, unpublished data). Therefore, using alternatives to copper and mancozeb early in the season for foliar pathogen management is advisable.

Ultraviolet reflective mulches also deter other pests, especially whiteflies and aphids, which can vector other plant viruses (Fanigliulo et al., 2009; Stapleton & Summers, 2002; Summers et al., 2010). Consequently, these materials are a good overall IPM tactic to employ where insect vectors are of concern. However, growers need to balance these benefits with the potential delay in plant growth in the spring because these mulches do not warm the soil as readily as standard black plastic mulches (Harpaz, 1982; Maynard & Olson, 2000). Newly developed mulches have helped to mitigate this effect by excluding the reflective metalized layer where transplants are placed.

5.6 Host plant fertilization

Soils in the southeastern USA tend to be nutrient deficient, so that growers need to add up to 200 kg of nitrogen per hectare (the recommended rate for Florida, Olson & Simmonne, 2009). However, growers have often overfertilized crops by up to 70% (Castro et al., 1993). This extra nitrogen fertilization can actually increase densities of western flower thrips. Female western flower thrips, in particular, preferentially settle on plants with higher nitrogen content (Baez et al., 2011; Brodbeck et al., 2001). This association seems to be most closely related to the phenylalanine content of tomato. From a pest management perspective, as vector populations increase with increasing fertilization there is an increase in the incidence of tomato spotted wilt. In north Florida tomatoes, the incidence of tomato spotted wilt was 50% lower for plants grown at recommended nitrogen levels compared with plants grown with supraoptimal nitrogen (Stavisky et al., 2002). Interestingly, *F. tritici* and *F. bispinosa* do not respond in the same manner to nitrogen fertilization as western flower thrips (Baez et al., 2011; Stavisky et al., 2002). Besides the increasing pest problems, excess nitrogen fertilization does not increase per plant yield. Therefore, growers can improve overall crop production of tomatoes and pepper by maintaining optimal fertilization levels.

5.7 Systemic acquired resistance

Many plants possess traits for systemic acquired resistance, which are induced defensive mechanisms against pathogens (Sticher et al., 1997). Certain chemicals have been found to stimulate these natural plant defenses against pathogens when applied to plants before infection occurs. Acibenzolar-*S*-methyl is a systemic acquired resistance inducer that stimulates the salicylic acid pathway for disease resistance in tomato and other crops. Commercial formulations of acibenzolar-*S*-methyl have been shown to reduce the incidence of tomato spotted wilt (Momol et al., 2004). Its use has minimal impacts on populations of the flower thrips. When tomatoes are grown on ultraviolet reflective mulches, Momol et al. (2004) concluded that acibenzolar-*S*-methyl provided little additional disease protection because of the large effect of the mulch. Nevertheless, acibenzolar-*S*-methyl is highly effective against bacterial pathogens that afflict tomatoes (Obradovic et al., 2005; Pradhanang et al., 2005), making it an excellent replacement for copper and mancozeb sprays on ultraviolet reflective mulches as well as standard black plastic mulch.

5.8 Host plant resistance

The single best defense against insect-vectored pathogens is host plant resistance. Numerous cultivars of tomato and pepper are resistant or tolerant to *Tomato spotted wilt virus* are currently commercially available (for a partial listing, see Funderburk et al., 2011). These cultivars have resistance to the virus, but not to thrips feeding or oviposition. All of the commercially available cultivars of tomato share a single source of resistance from the *Sw-5* gene. In pepper, all resistance is conferred by the *Tsw* gene. Both of the *Sw-5* and *Tsw* genes appear to be single dominant genes (Boiteux & de Avila, 1994; Stevens et al., 1992), and thus susceptible to being compromised by resistance breaking strains of the virus. In fact, such resistance breaking strains have commonly developed around the world (Roselló et al., 1996; Sharman & Persley, 2006). Presently in Florida, tomato spotted wilt resistant cultivars can maintain tomato spotted wilt incidences at economically acceptable levels. However, the threat of epidemics from resistance breaking strains is real. Coupled with the potential damage from western flower thrips feeding and oviposition, growers must maintain an integrated approach to thrips and tomato spotted wilt management.

5.9 Insecticides

Insecticides continue to have an important role to play in western flower thrips management. However, the use of insecticides must be done judiciously. Decisions regarding which insecticides to use and when need to be made in the context of both short-term and long-term management goals. Minimizing resistance development and avoiding the flaring of western flower thrips populations by their release from natural enemies need to be critical factors in insecticide use decisions. Populations of the invasive western flower thrips likely arrived in Florida with resistance to most classes of broad-spectrum insecticides (Immaraju et al., 1992). Further, flaring of the populations of the western flower thrips and other pests is possible when any broad-spectrum synthetic insecticide is used (Funderburk et al., 2000; Reitz et al., 2003). For this reason, growers are encouraged to move to newer, safer, and more selective insecticides in different chemical classes that are becoming available. Although growers are encouraged to use more selective materials when needed, the use of certain organophosphate and carbamate insecticides against western flower thrips may be warranted in certain circumstances. These should only be used in particular instances when nontarget effects would be minimal, for instance near the end of the production season to prevent scarring damage to fruit.

As discussed above, the most efficacious insecticides for western flower thrips, at present, are in the spinosyn class. No other insecticide class provides a similar level of effectiveness against western flower thrips. However, as resistance to spinosyns has been documented in Florida and elsewhere, limits are being placed on the number of applications that can be made in each crop to forestall further resistance development. A number of other insecticides are registered or in the process of being registered by the United States Environmental Protection Agency (EPA) that are able to suppress western flower thrips adults and larvae. Lists of currently available insecticides for western flower thrips management and their role in overall IPM programs for fruiting vegetable crops are available (Funderburk, 2009; Funderburk et al., 2011; Funderburk et al., 2009).

The fact that these materials are not as efficacious as spinosyns should not deter their inclusion in IPM programs. The focus of management should not be placed on killing the maximum number of thrips. Rather, the focus of management should be in minimizing damage below economically injurious levels. Because economic damage from oviposition

and scarring from feeding only occur at high levels (see above), even limited suppression of western flower thrips adults and larvae can maintain these types of damage well within tolerable limits. Secondary spread of tomato spotted wilt in tomato can also be limited by suppressing populations rather than attempting complete control (Momol et al., 2004). In pepper, conservation of *O. insidiosus* significantly reduces both primary and secondary spread of tomato spotted wilt (Funderburk et al., 2000; Reitz et al., 2003). We have found repeatedly that avoiding treatments that induce outbreaks of western flower thrips populations by killing natural enemies and competing species of native thrips within crop fields is an effective approach to minimizing losses to western flower thrips. Most broad-spectrum synthetic insecticides, including pyrethroids, organophosphates, and carbamates kill the native species of thrips that outcompete western flower thrips (Hansen et al., 2003; Reitz et al., 2003; Srivistava et al., 2008), leading to dramatic large scale shifts in thrips demographics (Frantz & Mellinger, 2009). These synthetic broad-spectrum insecticides not only can disrupt western flower thrips management, they also can disrupt management of other pests including spider mites, whiteflies, and leafminers, by eliminating natural enemies of those pests.

5.10 Vertical integration of the management program

One of the most important keys to successful crop production is not to consider problems in isolation. It is critical to understand how one management tactic may affect other production aspects. For example, in northern Florida, western flower thrips and *Tomato spotted wilt virus* are clearly the most important pest-complex facing tomato and pepper production. The use of ultraviolet reflective mulches has been effective in reducing populations of western flower thrips and the incidence of tomato spotted wilt (e.g., Momol et al., 2004; Reitz et al., 2003). Still, sweetpotato whitefly (*Bemisia tabaci* [Gennadius]) and whitefly-vectored viruses are occasionally important pests in northern Florida tomatoes (Momol et al., 1999). Ultraviolet reflective mulches used to manage thrips and tomato spotted wilt are also efficacious in reducing whitefly-caused damage (Antignus, 2000; Csizinszky et al., 1999).

In contrast, in southern Florida, western flower thrips and tospoviruses have only recently emerged as damaging problems requiring management consideration. Whiteflies and whitefly-vectored viruses have historically been the key insect pest and disease problems in tomato there. Growers use an wide range of insecticides to manage whitefly vectored viruses, particularly *Tomato yellow leaf curl virus*, which can devastate entire crops (Moriones & Navas-Castillo, 2000). Most tomatoes in southern Florida are treated with neonicotinoid insecticides at planting for management of immature whiteflies, with imidacloprid, thiamethoxam, or dinotefuran being most commonly used (Schuster et al., 2010). As the season progresses, growers may rotate "soft" insecticides, such as azadirachtin-based products, microbial insecticides such as *Beauveria bassiana* and insect growth regulators, into management programs against whitefly nymphs. While such materials are compatible with thrips management, they have little effect in suppressing primary virus transmission by whitefly adults that disperse into fields. As a result, growers still place a heavy reliance on broad spectrum insecticides, such as organophosphates and pyrethroids, for management of primary virus spread by whiteflies. The unintended consequence of this approach to whitefly management has been to release populations of western flower thrips from their natural controls, which greatly complicates overall crop management (Frantz & Mellinger, 2009; Weiss et al., 2009).

To facilitate overall crop management, growers are advised to anticipate key pests such as whiteflies or western flower thrips and to employ preventive tactics to minimize their impact. For tomato, one such preventative tactic would ultraviolet reflective mulches. It is also important from an areawide management perspective to maintain crop free periods and remove crops immediately after harvest so that crop residues do not serve as reservoirs for later infestations. In pepper grown in southern Florida, pepper weevil, *Anthonomus eugenii* Cano, is another significant pest. Its management also can be facilitated by crop free periods, and the destruction of crops immediately after harvest. Further sanitation, including the control of solanaceous weeds that serve as alternative hosts helps to reduce future populations. Using such preventative measures would minimize the need for insecticide applications for this pest. Consequently, there would be an overall benefit crop management because many of the available insecticides for pepper weevil management are pyrethroids or other disruptive broad spectrum synthetic insecticides. These are just some of the many pest problems that growers must contend with. Consequently, there is a clear need to integrate management programs for the diverse pests attacking crops. It is also critical to provide growers with realistic economic thresholds for different pests, and proper scouting techniques to assess pest abundances and the need, if any, to apply pesticides.

6. Conclusions and future directions

Only recently has the reliance on insecticides for western flower thrips management been challenged. Yet, failures to control western flower thrips with insecticides have become so severe that Cloyd (2009b) suggested we have reached an impasse in the use of insecticides against western flower thrips in ornamental greenhouse production and diverse management tactics must be employed. The successful management programs for western flower thrips and thrips-vectored viruses developed for solanaceous crops in Florida have been based on an understanding of thrips ecology and how different species interact with different crops (Funderburk, 2009). These strategies involve: an emphasis on scouting and the identification of thrips species present in a crop; the optimal nitrogen fertilization inputs to reduce the attractiveness of crops to western flower thrips without adversely affecting yields; the use of ultraviolet reflective mulches to deter thrips entrance into crop fields; the use of acibenzolar-*S*-methyl to suppress development of tomato spotted wilt symptoms in fruit of susceptible varieties; and the use of economic thresholds to determine the need and timing of insecticide applications; and the use of select insecticides to help suppress reproduction of thrips in the field, and thus manage secondary spread of tomato spotted wilt from within fields. The understanding of the importance of biotic resistance against western flower thrips provided by competing species of native thrips and natural enemies, such as *O. insidiosus,* has been of fundamental importance to improving western flower thrips management. This low input approach helps to avoid other pest problems as well. For example, the conservation biological control program for western flower thrips and *Tomato spotted wilt virus* in peppers has been used by growers in north Florida since the late 1990's, and growers have experienced far fewer problems from whiteflies, aphids, and other pests than when they were following a prophylactic, calendar-based spray program that included frequent use of broad-spectrum insecticides to control pests.

If western flower thrips were the only pest concern for growers, they would, perhaps, feel relieved. However, there are many other insect and pathogen threats to crops. We have now

begun to appreciate how management of one pest can interact with and affect management of other pests. Therefore, the need for sustainable, truly integrated pest management programs that do not consider individual pests in isolation is clear. There must be a focus on pest complexes rather than considering individual pest species. For example, insecticide resistance can be exacerbated when western flower thrips are exposed to insecticide treatments aimed at other pests (Immaraju et al., 1992).

The development of more selective insecticides that do not adversely affect natural enemies will improve overall crop management. These materials would reduce the risk of releasing non-target pests from control when treatments are necessary for another pest. Additional management tools that would reduce the need for insecticide applications will continue to be beneficial. Improved host plant resistance is one of those areas. The recent development of cultivars of tomatoes and peppers that show some degree of resistance to *Tomato spotted wilt virus* has eased concerns among growers. However, because the resistance conferred by the *Sw-5* gene in tomato and the *Tsw* gene in pepper is not durable, resistance-breaking strains can readily develop (Aramburu & Marti, 2003; Ciuffo et al., 2005; Latham & Jones, 1998; Margaria et al., 2004; Roggero et al., 2002; Thomas-Carroll & Jones, 2003; Thompson & van Zijl, 1995). In addition, virus resistance does not protect against physical damage. Therefore, it is still important for growers to maintain a multifaceted integrated management program.

Currently there are major research efforts underway to identify new germplasm sources of resistance to *Tomato spotted wilt virus*. One promising source of resistance appears to be the *Sw-7* gene from *Solanum chilense* (Price et al., 2007). Although this resistance is another single gene dominant trait, it is not linked to the *Sw-5* gene. Another exciting prospect for the future of host plant resistance is the identification of germplasm that is resistant to western flower thrips. Several accessions of different *Capsicum* species have been identified that have significant resistance against western flower thrips feeding (Maharijaya et al., 2011). If these traits can be incorporated into commercial cultivars, they may offer some degree of protection against virus transmission and aesthetic damage.

The management of western flower thrips will continue to be an ongoing challenge. Management programs cannot be static as they will need to be continually refined and updated with the advent of new invasive pests or as other conditions change. For example, *Scirtothrips dorsalis* Hood invaded Florida within the past five years and poses a threat to crops including pepper and eggplant. Recently, a new form of *Tospovirus,* a genetic reassortant of *Groundnut ringspot virus* and *Tomato chlorotic spot virus* has been found in Florida (Webster et al., 2010). Although western flower thrips can transmit this virus (Webster et al., 2011), it is not yet known if it or another thrips species is the most important vector. Despite these challenges, we believe that western flower thrips can be successfully managed, given a thorough understanding of its ecology and pest status.

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8. References

Antignus, Y. (2000) Manipulation of wavelength-dependent behaviour of insects: An IPM tool to impede insects and restrict epidemics of insect-borne viruses. *Virus Research* 71: 213-220.

Antignus, Y., Mor, N., Joseph, R.B., Lapidot, M., & Cohen, S. (1996) Ultraviolet-absorbing plastic sheets protect crops from insect pests and from virus diseases vectored by insects. *Environmental Entomology* 25: 919-924.

Aramburu, J., & Marti, M. (2003) The occurrence in north-east Spain of a variant of Tomato spotted wilt virus (TSWV) that breaks resistance in tomato *(Lycopersicon esculentum)* containing the Sw-5 gene. *Plant Pathology* 52: 407.

Baez, I., Reitz, S.R., & Funderburk, J.E. (2004) Predation by *Orius insidiosus* (Heteroptera: Anthocoridae) on species and life stages of *Frankliniella* flower thrips (Thysanoptera: Thripidae) in pepper flowers. *Environmental Entomology* 33: 662-670.

Baez, I., Reitz, S.R., Funderburk, J.E., & Olson, S.M. (2011) Variation within and between *Frankliniella* thrips species in host plant utilization. *Journal of Insect Science* 11: 41.

Bauske, E.M. (1998) Southeastern tomato growers adopt integrated pest management. *HortTechnology* 8: 40-44.

Beshear, R.J. (1983) New records of thrips in Georgia (Thysanoptera, Terebrantia, Tubulifera). *Journal of Georgia Entomological Society* 18: 342-344.

Bielza, P. (2008) Insecticide resistance management strategies against the western flower thrips, *Frankliniella occidentalis*. *Pest Management Science* 64: 1131-1138.

Bielza, P., Espinosa, P.J., Quinto, V., Abellán, J., & Contreras, J. (2007a) Synergism studies with binary mixtures of pyrethroid, carbamate and organophosphate insecticides on *Frankliniella occidentalis* (Pergande). *Pest Management Science* 63: 84-89.

Bielza, P., Quinto, V., Contreras, J., Torne, M., Martin, A., & Espinosa, P.J. (2007b) Resistance to spinosad in the western flower thrips, *Frankliniella occidentalis* (Pergande), in greenhouses of south-eastern Spain. *Pest Management Science* 63: 682-687.

Bielza, P., Quinto, V., Fernandez, E., Gravalos, C., & Contreras, J. (2007c) Genetics of spinosad resistance in *Frankliniella occidentalis* (Thysanoptera: Thripidae). *Journal of Economic Entomology* 100: 916-920.

Bielza, P., Quinto, V., Grávalos, C., Abellán, J., & Fernández, E. (2008) Lack of fitness costs of insecticide resistance in the western flower thrips (Thysanoptera: Thripidae). *Journal of Economic Entomology* 101: 499-503.

Bohart, R.M. (1943) Calcium cyanide fumigation for the western thrips. *Journal of Economic Entomology* 36: 442-444.

Boiteux, L.S., & de Avila, A.C. (1994) Inheritance of a resistance specific to tomato spotted wilt Tospovirus in *Capsicum chinense* 'PI 159236'. *Euphytica* 75: 139-142.

Bonarriva, J. (2003) *Cut Flowers: Industry and Trade Summary*, United States International Trade Commission, Available from: http://www.usitc.gov/publications/332/pub3580.pdf.

Borden, A.D. (1915) The mouthparts of the Thysanoptera and the relation of thrips to the non-setting of certain fruits and seeds. *Journal of Economic Entomology* 8: 354-360.

Bostanian, N.J., Akalach, M., & Chiasson, H. (2005) Effects of a *Chenopodium*-based botanical insecticide/acaricide on *Orius insidiosus* (Hemiptera: Anthocoridae) and *Aphidius colemani* (Hymenoptera: Braconidae). *Pest Management Science* 61: 979-984.

Broadbent, A.B., & Pree, D.J. (1997) Resistance to insecticides in populations of *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) from greenhouses in the Niagara region of Ontario. *Canadian Entomologist* 129: 907-913.

Brodbeck, B.V., Stavisky, J., Funderburk, J.E., Andersen, P.C., & Olson, S.M. (2001) Flower nitrogen status and populations of *Frankliniella occidentalis* feeding on *Lycopersicon esculentum*. *Entomologia Experimentalis et Applicata* 99: 165-172.

Brødsgaard, H.F. (1993) Cold hardiness and tolerance to submergence in water in *Frankliniella occidentalis* (Thysanoptera: Thripidae). *Environmental Entomology* 22: 647-653.

Brødsgaard, H.F. (1994) Insecticide resistance in European and African strains of western flower thrips (Thysanoptera: Thripidae) tested in a new residue-on-glass test. *Journal of Economic Entomology* 87: 1141-1146.

Broughton, S., & Herron, G.A. (2007) *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) chemical control: insecticide efficacy associated with the three consecutive spray strategy. *Australian Journal of Entomology* 46: 140-145.

Broughton, S., & Herron, G.A. (2009) Potential new insecticides for the control of western flower thrips (Thysanoptera: Thripidae) on sweet pepper, tomato, and lettuce. *Journal of Economic Entomology* 102: 646-651.

Bryan, D.E., & Smith, R.F. (1956) The *Frankliniella occidentalis* (Pergande) complex in California (Thysanoptera: Thripidae). *University of California Publications in Entomology* 10: 359-410.

Butt, T.M., & Brownbridge, M. (1997) Fungal pathogens of thrips, In: T. Lewis (Ed.), *Thrips as crop pests*, CAB International, New York. pp. 399-433.

Castro, B.F., Durden, B.R., Olson, S.M., & Rhoads, F.M. (1993) Telogia Creek irrigation energy conservation demonstration on mulched staked tomatoes. *Proceedings of the Florida State Horticultural Society* 106: 219-222.

Chiasson, H., Vincent, C., & Bostanian, N.J. (2004) Insecticidal properties of a *Chenopodium*-based botanical. *Journal of Economic Entomology* 97: 1378-1383.

Childers, C.C. (1997) Feeding and oviposition injuries to plants, In: T. Lewis (Ed.), *Thrips as crop pests*, CAB International, New York. pp. 505-537.

Childs, L. (1927) Two Species of Thrips injurious to Apples in the Pacific Northwest. *Journal of Economic Entomology* 20: 805-808 pp.

Ciuffo, M., Finetti-Sialer, M.M., Gallitelli, D., & Turina, M. (2005) First report in Italy of a resistance-breaking strain of *Tomato spotted wilt virus* infecting tomato cultivars carrying the *Sw5* resistance gene. *Plant Pathology* 54: 564.

Cloyd, R.A. (2009a) Getting mixed-up: Are greenhouse producers adopting appropriate pesticide mixtures to manage arthropod pests? *HortTechnology* 19: 638-646.

Cloyd, R.A. (2009b) Western flower thrips *(Frankliniella occidentalis)* management on ornamental crops grown in greenhouses: Have we reached an impasse? *Pest Technology* 3: 1-9.

Cloyd, R.A., Galle, C.L., Keith, S.R., Kalscheur And, N.A., & Kemp, K.E. (2009) Effect of commercially available plant-derived essential oil products on arthropod pests. *Journal of Economic Entomology* 102: 1567-1579.

Cloyd, R.A., & Sadof, C.S. (1998) Flower quality, flower number, and western flower thrips density on transvaal daisy treated with granular insecticides. *HortTechnology* 8: 567-570.

Cook, D.F., Dadour, I.R., & Bailey, W.J. (2002) Addition of alarm pheromone to insecticides and the possible improvement of the control of the western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae). *International Journal of Pest Management* 48: 287-290.

Coutts, B.A., & Jones, R.A.C. (2005) Suppressing spread of Tomato spotted wilt virus by drenching infected source or healthy recipient plants with neonicotinoid insecticides to control thrips vectors. *Annals of Applied Biology* 146: 95-103.

Crawford, D.L. (1915) Potato curly leaf (caused by *Euthrips occidentalis*). *California State Commission of Horticulture, Monthly Bulletin* 4: 389-391, Number 8, August.

Csinos, A., Martinez-Ochoa, N., & Bertrand, P. (2009) History, In: S.W. Mullis (Ed.), *Tospoviruses in Solanaceae and Other Crops in the Coastal Plain of Georgia*, University of Georgia, Athens, GA. pp. 7-8.

Csizinszky, A.A., Schuster, D.J., & Polston, J.E. (1999) Effect of ultraviolet-reflective mulches on tomato yields and on the silverleaf whitefly. *Hortscience* 34: 911-914.

Dağli, F., & Tunç, I. (2007) Insecticide resistance in *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) collected from horticulture and cotton in Turkey. *Australian Journal of Entomology* 46: 320-324.

Dağlı, F., & Tunç, I. (2008) Insecticide resistance in *Frankliniella occidentalis*: Corroboration of laboratory assays with field data and cross-resistance in a cypermethrin-resistant strain. *Phytoparasitica* 36: 352-359.

Daughtrey, M.L., Jones, R.K., Moyer, J.W., Daub, M.E., & Baker, J.R. (1997) Tospoviruses strike the greenhouse industry: INSV has become a major pathogen on flower crops. *Plant Disease* 81: 1220-1230.

Denholm, I., Cahill, M., Dennehy, T.J., & Horowitz, A.R. (1998) Challenges with managing insecticide resistance in agricultural pests, exemplified by the whitefly *Bemisia tabaci*. *Philosophical Transactions of the Royal Society of London Series B Biological Sciences* 353: 1757-1767.

Dripps, J.E., Gomez, L.E., Weiss, A.W., Funderburk, J., Castro, B.A., & Paroonagian, D.L. (2010) Insecticide rotation as a component of thrips resistance management programs. *Resistant Pest Management Newsletter* 19: 32-35.

Eger, J.E., Stavisky, J., & Funderburk, J.E. (1998) Comparative toxicity of spinosad to *Frankliniella* spp. (Thysanoptera: Thripidae), with notes on a bioassay technique. *Florida Entomologist* 81: 547-551.

Espinosa, P.J., Bielza, P., Contreras, J., & Lacasa, A. (2002a) Field and laboratory selection of *Frankliniella occidentalis* (Pergande) for resistance to insecticides. *Pest Management Science* 58: 920-927.

Espinosa, P.J., Bielza, P., Contreras, J., & Lacasa, A. (2002b) Insecticide resistance in field populations of *Frankliniella occidentalis* (Pergande) in Murcia (south-east Spain). *Pest Management Science* 58: 967-971.

Espinosa, P.J., Contreras, J., Quinto, V., Grávalos, C., Fernández, E., & Bielza, P. (2005) Metabolic mechanisms of insecticide resistance in the western flower thrips, *Frankliniella occidentalis* (Pergande). *Pest Management Science* 61: 1009-1015.

Essig, E.O. (1926) *Insects of Western North America* Macmillan, New York.

Fanigliulo, A., Comes, S., Pacella, R., Crescenzi, A., Momol, M.T., Olson, S.M., & Reitz, S. (2009) Integrated management of viral diseases in field-grown tomatoes in southern Italy. *Acta Horticulturae* 808: 387-392.

Frantz, G., & Fasulo, T.R. (n.d.) Thrips KnowledgeBase, Accessed: 20 June 2011, Available from: http://www.gladescropcare.com/pg1.html.

Frantz, G., & Mellinger, H.C. (2009) Shifts in western flower thrips, *Frankliniella occidentalis* (Thysanoptera: Thripidae), population abundance and crop damage. *Florida Entomologist* 92: 29-34.

Funderburk, J. (2009) Management of the western flower thrips (Thysanoptera: Thripidae) in fruiting vegetables. *Florida Entomologist* 92: 1-6.

Funderburk, J., Reitz, S., Olson, S., Stansly, P., Smith, H., McAvoy, G., Demirozer, O., Snodgrass, C., Paret, M., & Leppla, N. (2011) Managing thrips and tospoviruses in tomato, University of Florida, Gainesville, FL.

Funderburk, J., Reitz, S., Stansly, P., Schuster, D., Nuessly, G., & Leppla, N. (2009) Managing thrips in pepper and eggplant, ENY-658, Accessed: 26 July 2011, Available from: http://edis.ifas.ufl.edu.

Funderburk, J., Stavisky, J., & Olson, S. (2000) Predation of *Frankliniella occidentalis* (Thysanoptera: Thripidae) in field peppers by *Orius insidiosus* (Hemiptera: Anthocoridae). *Environmental Entomology* 29: 376-382.

Georghiou, G.P., & Taylor, C.E. (1986) Factors influencing the evolution of resistance, In: Committee on Strategies for the Management of Pesticide Resistant Pest Populations: National Research Council (Ed.), *Pesticide resistance: strategies and tactics for management*, National Academies Press, Washington, DC. pp. 157-169.

Ghidiu, G.M., Hitchner, E.M., & Funderburk, J.E. (2006) Goldfleck damage to tomato fruit caused by feeding of *Frankliniella occidentalis* (Thysanoptera: Thripidae). *Florida Entomologist* 89: 279-281.

Gitaitis, R.D., Dowler, C.C., & Chalfant, R.B. (1998) Epidemiology of tomato spotted wilt in pepper and tomato in southern Georgia. *Plant Disease* 82: 752-756.

Goldbach, R., & Peters, D. (1994) Possible causes of the emergence of tospovirus diseases. *Seminars in Virology* 5: 113-120.

Hale, R.L., & Shorey, H.H. (1965) Systemic insecticides for the control of western flower thrips on bulb onions. *Journal of Economic Entomology* 58: 793-794.

Hansen, E.A., Funderburk, J.E., Reitz, S.R., Ramachandran, S., Eger, J.E., & McAuslane, H. (2003) Within-plant distribution of *Frankliniella* species (Thysanoptera: Thripidae)

and *Orius insidiosus* (Heteroptera: Anthocoridae) in field pepper. *Environmental Entomology* 32: 1035-1044.

Harding, J.A. (1961a) Effect of migration, temperature, and precipitation on thrips infestations in south Texas. *Journal of Economic Entomology* 54: 77-79.

Harding, J.A. (1961b) Studies on the control of thrips attacking onions. *Journal of Economic Entomology* 54: 1254-1255.

Harpaz, I. (1982) Nonpesticidal control of vector-borne viruses, In: K.F. Harris, & K. Maramorosch (Eds.), *Pathogens, Vectors and Plant Diseases: Approaches to Control*, Academic Press, New York. pp. 1-21.

Herron, G.A., & Cook, D.F. (2002) Initial verification of the resistance management strategy for *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) in Australia. *Australian Journal of Entomology* 41: 187-191.

Herron, G.A., & Gullick, G.C. (2001) Insecticide resistance in Australian populations of *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) causes the abandonment of pyrethroid chemicals for its control. *General and Applied Entomology* 30: 21-26.

Herron, G.A., & James, T.M. (2005) Monitoring insecticide resistance in Australian *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) detects fipronil and spinosad resistance. *Australian Journal of Entomology* 44: 299-303.

Huang, S.W. (2004) *Global Trade Patterns in Fruits and Vegetables*, USDA Economic Research Service, Available from: http://www.ers.usda.gov/publications/WRS0406/WRS0406.pdf.

Immaraju, J.A., Paine, T.D., Bethke, J.A., Robb, K.L., & Newman, J.P. (1992) Western flower thrips (Thysanoptera: Thripidae) resistance to insecticides in coastal California greenhouses. *Journal of Economic Entomology* 85: 9-14.

IRAC International MoA Working Group. (2011) IRAC MoA (Insecticide Resistance Action Committee Mode of Action) Classification Scheme, version 7.1, June 2011, Accessed: 3 July 2011, Available from: http://www.irac-online.org/wp-content/uploads/2009/09/MoA_Classification.pdf.

Isayama, S., Saito, S., Kuroda, K., Umeda, K., & Kasamatsu, K. (2005) Pyridalyl, a novel insecticide: Potency and insecticidal selectivity. *Archives of Insect Biochemistry and Physiology* 58: 226-233.

Ishaaya, I., Kontsedalov, S., & Horowitz, A.R. (2002) Emamectin, a novel insecticide for controlling field crop pests. *Pest Management Science* 58: 1091-1095.

Jacobson, A.L., & Kennedy, G.G. (2011) The effect of three rates of cyantraniliprole on the transmission of tomato spotted wilt virus by *Frankliniella occidentalis* and *Frankliniella fusca* (Thysanoptera: Thripidae) to *Capsicum annuum*. *Crop Protection* 30: 512-515.

Jensen, F. (1973) Timing of halo spotting by flower thrips on table grapes. *California Agriculture* 31: 6-8.

Jensen, S.E. (1998) Acetylcholinesterase activity associated with methiocarb resistance in a strain of western flower thrips, *Frankliniella occidentalis* (Pergande). *Pesticide Biochemistry and Physiology* 61: 191-200.

Jensen, S.E. (2000a) Insecticide resistance in the western flower thrips, *Frankliniella occidentalis*. *Integrated Pest Management Reviews* 5: 131-146.

Jensen, S.E. (2000b) Mechanisms associated with methiocarb resistance in *Frankliniella occidentalis* (Thysanoptera: Thripidae). *Journal of Economic Entomology* 93: 464-471.

Joost, P.H., & Riley, D.G. (2005) Imidacloprid effects on probing and settling behavior of *Frankliniella fusca* and *Frankliniella occidentalis* (Thysanoptera: Thripidae) in tomato. *Journal of Economic Entomology* 98: 1622-1629.

Kay, I.R., & Herron, G.A. (2010) Evaluation of existing and new insecticides including spirotetramat and pyridalyl to control *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) on peppers in Queensland. *Australian Journal of Entomology* 49: 175-181.

Kirk, W.D.J. (1997a) Distribution, abundance and population dynamics, In: T. Lewis (Ed.), *Thrips as crop pests*, CAB International, New York. pp. 217-257.

Kirk, W.D.J. (1997b) Feeding, In: T. Lewis (Ed.), *Thrips as crop pests*, CAB International, New York. pp. 119-174.

Kirk, W.D.J., & Terry, L.I. (2003) The spread of the western flower thrips *Frankliniella occidentalis* (Pergande). *Agricultural and Forest Entomology* 5: 301-310.

Kontsedalov, S., Weintraub, P.G., Horowitz, A.R., & Ishaaya, I. (1998) Effects of insecticides on immature and adult western flower thrips (Thysanoptera: Thripidae) in Israel. *Journal of Economic Entomology* 91: 1067-1071.

Latham, L.J., & Jones, R.A.C. (1998) Selection of resistance breaking strains of tomato spotted wilt tospovirus. *Annals of Applied Biology* 133: 385-402.

Maharijaya, A., Vosman, B., Steenhuis-Broers, G., Harpenas, A., Purwito, A., Visser, R.G.F., & Voorrips, R.E. (2011) Screening of pepper accessions for resistance against two thrips species (*Frankliniella occidentalis* and *Thrips parvispinus*). *Euphytica*: 1-10.

Mantel, W.P., & Van de Vrie, M. (1988) The western flower thrips, *Frankliniella occidentalis*, a new thrips species causing damage in protected cultures in The Netherlands. *Entomologische Berichten (Amsterdam)* 48: 140-142.

Margaria, P., Ciuffo, M., & Turina, M. (2004) Resistance breaking strain of *Tomato spotted wilt virus* (*Tospovirus; Bunyaviridae*) on resistant pepper cultivars in Almería, Spain. *Plant Pathology* 53: 795-795.

Martin, N.A., & Workman, P.J. (1994) Confirmation of a pesticide-resistant strain of Western flower thrips in New Zealand. *Proceedings of the NZ Plant Protection Conference* 47: 144-148.

Matteson, N., Terry, I., Ascoli-Christensen, A., & Gilbert, C. (1992) Spectral efficiency of the western flower thrips, *Frankliniella occidentalis*. *Journal of Insect Physiology* 38: 453-459.

Maymó, A.C., Cervera, A., Garcerá, M.D., Bielza, P., & Martínez-Pardo, R. (2006) Relationship between esterase activity and acrinathrin and methiocarb resistance in field populations of western flower thrips, *Frankliniella occidentalis*. *Pest Management Science* 62: 1129-1137.

Maynard, D.N., & Olson, S.M. (2000) *Vegetable production guide for Florida* University of Florida, IFAS, Florida Cooperative Extension Service, Gainesville, FL.

McDonald, J.R., Bale, J.S., & Walters, K.F.A. (1997) Low temperature mortality and overwintering of the western flower thrips *Frankliniella occidentalis* (Thysanoptera: Thripidae). *Bulletin of Entomological Research* 87: 497-505.

Momol, M., Simone, G., Dankers, W., Sprenkel, R., Olson, S., Momol, E., Polston, J., & Hiebert, E. (1999) First report of tomato yellow leaf curl virus in tomato in South Georgia. *Plant Disease* 83: 487-487.

Momol, M.T., Olson, S.M., Funderburk, J.E., Stavisky, J., & Marois, J.J. (2004) Integrated management of tomato spotted wilt on field-grown tomatoes. *Plant Disease* 88: 882-890.

Moriones, E., & Navas-Castillo, J. (2000) Tomato yellow leaf curl virus, an emerging virus complex causing epidemics worldwide. *Virus Research* 71: 123-134.

Morishita, M. (2001) Toxicity of some insecticides to larvae of *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) evaluated by the petri dish-spraying tower method. *Applied Entomology and Zoology* 36: 137-141.

Moulton, D. (1931) Western Thysanoptera of economic importance. *Journal of Economic Entomology* 24: 1031-1036.

Mound, L.A. (2011) Thysanoptera (Thrips) of the World – a checklist, Accessed: 26 July 2011, Available from: http://www.ento.csiro.au/thysanoptera/worldthrips.html.

Naegele, J.A., & Jefferson, R.N. (1964) Floricultural entomology. *Annual Review of Entomology* 9: 319-340.

Nickle, D.A. (2004) Commonly intercepted thrips (Thysanoptera) from Europe, the Mediterranean, and Africa at U.S. ports-of-entry. Part II. *Frankliniella* Karny and *Iridothrips* Priesner (Thripidae). *Proceedings of the Entomological Society of Washington* 106: 438-452.

Northfield, T.D., Paini, D.R., Funderburk, J.E., & Reitz, S.R. (2008) Annual cycles of *Frankliniella* spp. (Thysanoptera: Thripidae) thrips abundance on north Florida uncultivated reproductive hosts: Predicting possible sources of pest outbreaks. *Annals of the Entomological Society of America* 101: 769-778.

Obradovic, A., Jones, J.B., Momol, M.T., Olson, S.M., Jackson, L.E., Balogh, B., Guven, K., & Iriarte, F.B. (2005) Integration of biological control agents and systemic acquired resistance inducers against bacterial spot on tomato. *Plant Disease* 89: 712-716.

Olson, S.M., & Funderburk, J.E. (1986) A new threatening pest in Florida - Western flower thrips, In: W.M. Stall (Ed.), *Proceedings of the Florida Tomato Institute*, University of Florida Extension Report, Gainesville, FL. pp. 43-51.

Olson, S.M., & Simmonne, E. (2009) *Vegetable Production Handbook for Florida*, Univ. of Florida, IFAS, Florida Cooperative Extension Service, Gainesville, FL. pp. 247.

Paini, D.R., Funderburk, J.E., Jackson, C.T., & Reitz, S.R. (2007) Reproduction of four thrips species (Thysanoptera: Thripidae) on uncultivated hosts. *Journal of Entomological Science* 42: 610-615.

Paini, D.R., Funderburk, J.E., & Reitz, S.R. (2008) Competitive exclusion of a worldwide invasive pest by a native. Quantifying competition between two phytophagous insects on two host plant species. *Journal of Animal Ecology* 77: 184-190.

Pappu, H.R., Jones, R.A.C., & Jain, R.K. (2009) Global status of tospovirus epidemics in diverse cropping systems: Successes achieved and challenges ahead. *Virus Research* 141: 219-236.

Pearsall, I.A., & Myers, J.H. (2001) Spatial and temporal patterns of dispersal of western flower thrips (Thysanoptera: Thripidae) in nectarine orchards in British Columbia. *Journal of Economic Entomology* 94: 831-843.

Pergande, T. (1895) Observations on certain Thripidae. *Insect Life* 7: 390 – 395

Pfannenstiel, R.S., & Yeargan, K.V. (1998) Association of predaceous Hemiptera with selected crops. *Environmental Entomology* 27: 232-239.

Pradhanang, P.M., Ji, P., Momol, M.T., Olson, S.M., Mayfield, J.L., & Jones, J.B. (2005) Application of acibenzolar-S-methyl enhances host resistance in tomato against *Ralstonia solanacearum*. *Plant Disease* 89: 989-993.

Price, D.L., Memmott, F.D., Scott, J.W., Olson, S.M., & Steven, M.R. (2007) Identification of molecular markers linked to a new *Tomato spotted wilt virus* resistance source in tomato. *Tomato Genetics Cooperative* 57: 35-36, Available from: http://tgc.ifas.ufl.edu/vol57/vol57.pdf.

Price, R.G., Walton, R.R., & Drew, W.A. (1961) Arthropod pests collected in Oklahoma greenhouses. *Journal of Economic Entomology* 54: 819-821.

Puche, H., Berger, R.D., & Funderburk, J.E. (1995) Population dynamics of *Frankliniella* species (Thysanoptera: Thripidae) thrips and progress of spotted wilt in tomato fields. *Crop Protection* 14: 577-583.

Race, S.R. (1961) Early-season thrips control on cotton in New Mexico. *Journal of Economic Entomology* 54: 974-976.

Race, S.R. (1965) Predicting thrips populations on seedling cotton. *Journal of Economic Entomology* 58: 1013-1014.

Ramachandran, S., Funderburk, J., Stavisky, J., & Olson, S. (2001) Population abundance and movement of *Frankliniella* species and *Orius insidiosus* in field pepper. *Agricultural and Forest Entomology* 3: 1-10.

Reitz, S.R. (2009) Biology and ecology of the western flower thrips (Thysanoptera: Thripidae): The making of a pest. *Florida Entomologist* 92: 7-13.

Reitz, S.R., Funderburk, J.E., & Waring, S.M. (2006) Differential predation by the generalist predator *Orius insidiosus* on congeneric species of thrips that vary in size and behavior. *Entomologia Experimentalis et Applicata* 119: 179-188.

Reitz, S.R., Gao, Y.L., & Lei, Z.R. (2011) Thrips: Pests of concern to China and the United States. *Agricultural Sciences in China* 10: 867-892.

Reitz, S.R., Maiorino, G., Olson, S., Sprenkel, R., Crescenzi, A., & Momol, M.T. (2008) Integrating plant essential oils and kaolin for the sustainable management of thrips and tomato spotted wilt on tomato. *Plant Disease* 92: 878-886.

Reitz, S.R., Yearby, E.L., Funderburk, J.E., Stavisky, J., Momol, M.T., & Olson, S.M. (2003) Integrated management tactics for *Frankliniella* thrips (Thysanoptera: Thripidae) in field-grown pepper. *Journal of Economic Entomology* 96: 1201-1214.

Riherd, P.T. (1942) An unusual injury to peas by thrips. *Journal of Economic Entomology* 35: 453-453.

Robb, K.L. (1989) *Analysis of Frankliniella occidentalis (Pergande) as a pest of floricultural crops in California greenhouses*, University of California, Riverside, Riverside, CA. pp. 135.

Robb, K.L., Newman, J., Virzi, J.K., & Parella, M.P. (1995) Insecticide resistance in western flower thrips, In: B.L. Parker, M. Skinner, & T. Lewis (Eds.), *Thrips biology and management, NATO ASI series. Series A, Life sciences ; v. 276.*, Plenum Press, New York. pp. 341-346.

Roggero, P., Masenga, V., & Tavella, L. (2002) Field isolates of *Tomato spotted wilt virus* overcoming resistance in pepper and their spread to other hosts in Italy. *Plant Disease* 86: 950-954.

Roselló, S., Díez, M.J., & Nuez, F. (1996) Viral diseases causing the greatest economic losses to the tomato crop. I. The tomato spotted wilt virus - A review. *Scientia Horticulturae* 67: 117-150.

Rosenheim, J.A., Johnson, M.W., Mau, R.F.L., Welter, S.C., & Tabashnik, B.E. (1996) Biochemical preadaptations, founder events, and the evolution of resistance in arthropods. *Journal of Economic Entomology* 89: 263-273.

Rugman-Jones, P.F., Hoddle, M.S., & Stouthamer, R. (2010) Nuclear-mitochondrial barcoding exposes the global pest Western flower thrips (Thysanoptera: Thripidae) as two sympatric cryptic species in its native California. *Journal of Economic Entomology* 103: 877-886.

Sakimura, K. (1961) Field observations on the thrips vector species of the tomato spotted wilt virus in the San Pablo area, California. *Plant Disease Reporter* 45: 772-776.

Sakimura, K. (1962) *Frankliniella occidentalis* (Thysanoptera: Thripidae), a vector of the tomato spotted wilt virus, with special reference to the color forms. *Annals of the Entomological Society of America* 55: 387-389.

Salguero-Navas, V.E., Funderburk, J.E., Olson, S.M., & Beshear, R.J. (1991) Damage to tomato fruit by the western flower thrips (Thysanoptera: Thripidae). *Journal of Entomological Science* 26: 436-442.

Schuster, D.J., Mann, R.S., Toapanta, M., Cordero, R., Thompson, S., Cyman, S., Shurtleff, A., & Morris, R.F. (2010) Monitoring neonicotinoid resistance in biotype B of *Bemisia tabaci* in Florida. *Pest Management Science* 66: 186-195.

Seamaxs, H.L. (1923) The alfalfa thrips and its effect on alfalfa seed production. *Canadian Entomologist* 4: 101-105.

Seaton, K.A., Cook, D.F., & Hardie, D.C. (1997) The effectiveness of a range of insecticides against western flower thrips (*Frankliniella occidentalis*) (Thysanoptera: Thripidae) on cut flowers. *Australian Journal of Agricultural Research* 48: 781-787.

Shah, P.A., & Goettel, M.S. (1999) *Directory of microbial control products and services* Society for Invertebrate Pathology, Gainseville, Florida, USA.

Sharman, M., & Persley, D.M. (2006) Field isolates of Tomato spotted wilt virus overcoming resistance in capsicum in Australia. *Australasian Plant Pathology* 35: 123-128.

Shorey, H.H., & Hall, I.M. (1962) Effect of chemical and microbial insecticides on several insect pests of lettuce in southern California. *Journal of Economic Entomology* 55: 169-174.

Shorey, H.H., & Hall, I.M. (1963) Toxicity of chemical and microbial insecticides to pest and beneficial insects on poled tomatoes. *Journal of Economic Entomology* 56: 813-817.

Shorey, H.H., Reynolds, H.T., & Anderson, L.D. (1962) Effect of Zectran, Sevin, and other new carbamate insecticides upon insect populations found on vegetable and field crops in southern California. *Journal of Economic Entomology* 55: 6-11.

Sparks, A.N., Jr. (2003)Vegetable pests, In: *Summary of Losses from Insect Damage and Cost of Control in Georgia - 2003*, P. Guillebeau, N. Hinkle, & P. Roberts (Eds.), pp. 41-47, Department of Entomology, University of Georgia, Retrieved from http://www.ent.uga.edu/pubs/SurveyLoss03.pdf.

Sparks, A.N., Jr. (2004)Vegetable pests, In: *Summary of Losses from Insect Damage and Cost of Control in Georgia - 2004*, P. Guillebeau, N. Hinkle, & P. Roberts (Eds.), pp. 42-48, Department of Entomology, University of Georgia, Retrieved from http://www.ent.uga.edu/pubs/SurveyLoss04.pdf.

Sparks, A.N., Jr. (2005)Vegetable pests, In: *Summary of Losses from Insect Damage and Cost of Control in Georgia - 2005*, P. Guillebeau, N. Hinkle, & P. Roberts (Eds.), pp. 31-37, Department of Entomology, University of Georgia, Retrieved from http://www.ent.uga.edu/pubs/SurveyLoss05.pdf.

Sparks, A.N., Jr. (2006)Vegetable pests, In: *Summary of Losses from Insect Damage and Cost of Control in Georgia - 2006*, P. Guillebeau, N. Hinkle, & P. Roberts (Eds.), pp. 38-43, Department of Entomology, University of Georgia, Retrieved from http://www.ent.uga.edu/pubs/SurveyLoss06.pdf.

Sparks, A.N., Jr., & Riley, D. (2001)Vegetable pests, In: *Summary of Losses from Insect Damage and Cost of Control in Georgia - 2001*, P. Guillebeau, N. Hinkle, & P. Roberts (Eds.), pp. 42-47, Department of Entomology, University of Georgia, Retrieved from http://www.ent.uga.edu/pubs/SurveyLoss01.pdf.

Sparks, A.N., Jr., & Riley, D. (2002)Vegetable pests, In: *Summary of Losses from Insect Damage and Cost of Control in Georgia - 2002*, P. Guillebeau, N. Hinkle, & P. Roberts (Eds.), pp. 38-43, Department of Entomology, University of Georgia, Retrieved from http://www.ent.uga.edu/pubs/SurveyLoss02.pdf.

Sparks, T.C., Thompson, G.D., Kirst, H.A., Hertlein, M.B., Mynderse, J.S., Turner, J.R., & Worden, T.V. (1999) Fermentation-derived insect control agents, In: F. Hall, & J.J. Menn (Eds.), *Biopesticides: use and delivery*, Humana, Totowa, NJ. pp. 171-188.

Spiers, J.D., Davies Jr, F.T., He, C., Bográn, C.E., Heinz, K.M., Starman, T.W., & Chau, A. (2006) Effects of insecticides on gas exchange, vegetative and floral development, and overall quality of gerbera. *Hortscience* 41: 701-706.

Srivistava, M., Bosco, L., Funderburk, J., Olson, S., & Weiss, A. (2008) Spinetoram is compatible with the key natural enemy of *Frankliniella* species thrips in pepper. *Plant Health Progress* doi:10.1094/PHP-2008-0118-02-RS.

Stapleton, J.J., & Summers, C.G. (2002) Reflective mulches for management of aphids and aphid-borne virus diseases in late-season cantaloupe (*Cucumis melo L. var. cantalupensis*). *Crop Protection* 21: 891-898.

Stavisky, J., Funderburk, J.E., Brodbeck, B.V., Olson, S.M., & Andersen, P.C. (2002) Population dynamics of *Frankliniella* spp. and tomato spotted wilt incidence as influenced by cultural management tactics in tomato. *Journal of Economic Entomology* 95: 1216-1221.

Stevens, M.R., Scott, S.J., & Gergerich, R.C. (1992) Inheritance of a gene for resistance to *Tomato spotted wilt virus* Tswv from *Lycopersicon peruvianum* Mill. *Euphytica* 59: 9-17.

Sticher, L., Mauch-Mani, B., & Métraux, J.P. (1997) Systemic acquired resistance. *Annual Review of Phytopathology* 35: 235-270.

Summers, C.G., Newton, A.S., Mitchell, J.P., & Stapleton, J.J. (2010) Population dynamics of arthropods associated with early-season tomato plants as influenced by soil surface microenvironment. *Crop Protection* 29: 249-254.

Thalavaisundaram, S., Herron, G.A., Clift, A.D., & Rose, H. (2008) Pyrethroid resistance in *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) and implications for its management in Australia. *Australian Journal of Entomology* 47: 64-69.

Thomas-Carroll, M.L., & Jones, R.A.C. (2003) Selection, biological properties and fitness of resistance-breaking strains of Tomato spotted wilt virus in pepper. *Annals of Applied Biology* 142: 235-243.

Thompson, G.D., Dutton, R., & Sparks, T.C. (2000) Spinosad – a case study: an example from a natural products discovery programme. *Pest Management Science* 56: 696-702.

Thompson, G.J., & van Zijl, J.J.B. (1995) Control of Tomato spotted wilt virus in tomatoes in South Africa. *Acta Horticulturae* 431: 379-384.

US EPA. (1996) *Food Quality Protection Act of 1996, Public Law 104–170*, Available from: http://www.epa.gov/pesticides/regulating/laws/fqpa/gpogate.pdf.

Vacante, V., Cacciola, S.O., & Pennisi, A.M. (1994) Epizootiological study of *Neozygites parvispora* (Zygomycota: Entomophthoraceae) in a population of *Frankliniella occidentalis* (Thysanoptera: Thripidae) on pepper in Sicily. *Entomophaga* 39: 123-130.

van den Meiracker, R.A.F., & Ramakers, P.M.J. (1991) Biological control of the western flower thrips *Frankliniella ocidentalis* in sweet pepper with the anthocorid predator *Orius insidiosus*. *Mededelingen van de Faculteit Landbouwwetenschappen Rijksuniversiteit Gent* 56: 241-249.

Webster, C., Perry, K., Lu, X., Horsman, L., Frantz, G., Mellinger, C., & Adkins, S. (2010) First report of *Groundnut ringspot virus* infecting tomato in south Florida. *Plant Health Progress* 10.1094/PHP-2010-0707-01-BR.

Webster, C.G., Reitz, S.R., Perry, K.L., & Adkins, S. (2011) A natural M RNA reassortant arising from two species of plant- and insect-infecting bunyaviruses and comparison of its sequence and biological properties to parental species. *Virology* 413: 216-225.

Weiss, A., Dripps, J.E., & Funderburk, J. (2009) Assessment of implementation and sustainability of integrated pest management programs. *Florida Entomologist* 92: 24-28.

Wijkamp, I., Van De Wetering, F., Goldbach, R., & Peters, D. (1996) Transmission of tomato spotted wilt virus by *Frankliniella occidentalis:* median acquisition and inoculation access period. *Annals of Applied Biology* 129: 303-313.

Woglum, R.S., & Lewis, H.C. (1935) Notes on citrus pests new or seldom injurious in California. *Journal of Economic Entomology* 28: 1018-1021.

Yokoyama, V.Y. (1977) *Frankliniella occidentalis* and scars on table grapes. *Environmental Entomology* 6: 25-30.

Zhang, S.-Y., Kono, S., Murai, T., & Miyata, T. (2008) Mechanisms of resistance to spinosad in the western flower thrip, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae). *Insect Science* 15: 125-132.

Zhang, S.H., Lei, Z.R., Fan, S.Y., & Wen, J.Z. (2009) Pathogenicity of four *Beauveria bassiana* strains against *Frankliniella occidentalis* at different temperatures. *Plant Protection* 35: 64-67.

Zhao, G., Liu, W., Brown, J.M., & Knowles, C.O. (1995) Insecticide resistance in field and laboratory strains of western flower thrips (Thysanoptera: Thripidae). *Journal of Economic Entomology* 88: 1164-1170.

Zhao, G., Liu, W., & Knowles, C.O. (1994) Mechanisms associated with diazinon resistance in western flower thrips. *Pesticide Biochemistry and Physiology* 49: 13-23.

17

The Past and Present of Pear Protection Against the Pear Psylla, *Cacopsylla pyri* L.

Stefano Civolani
Department of Biology and Evolution, University of Ferrara, Ferrara, Agricultural Foundation "Fratelli Navarra", Malborghetto di Boara (Ferrara), Italy

1. Introduction

The pear psylla, *Cacopsylla pyri* L. (Hemiptera Psyllidae), is known in Europe for its extended infestations which may cause heavy economical losses to most pear growing regions. Pear (*Pyrus sp.* L.) is the second most relevant fruit species of temperate regions: the first one is apple (*Malus domestica* L.) and the third one is peach (*Prunus persica* L.). The top three world regions for pear production are China, Europe and North America. Total European pear production is presently stable around 2.6 million tones, according to 2010 data from the Fruit and Vegetable Service Center (CSO, Ferrara, Italy). In Europe, Italy and Spain are the largest producers, respectively with 35% and 20% of the total production. Pear production in France is decreasing (8%), mainly as a result of fireblight on "Passe Crassane", a highly susceptible variety. Until 2007, in the Netherlands and Belgium there was an increase in production with extensive planting of the "Conference" cultivar, but the first signs of saturation of the "Conference" market started to appear in 2007. The total pear production of the Netherlands and Belgium presently amounts to 9%.
All commercial varieties in Europe belong to the species *Pyrus communis* L. and they are all susceptible to *C. pyri*. The susceptibility increases when orchard techniques are aimed to maximize fruit production, such as the high density of plants per hectare, the large use of fertilizers and the intense irrigation (Fig. 1).

2. Damages caused by *C. pyri*

The damages that *C. pyri* may induce to pear trees are classified in two main types: 1) direct damages, weakening the plant by subtraction of nutrients; when the pest attack is intense, the plant wastes away with reduced production; 2) indirect damages, due to the production of a large amount of honeydew on which sooty molds develop (russetting fruits, Fig. 2), and also to the possible transmission of phytoplasms (Fig. 3).
In the first case the most damaging stages are the nymphs of all instars because of the high amount of honeydew (produced especially in spring and summer) dripping on everything including fruits. Besides lowering the fruit market value, honeydew favours the growth of sooty molds caused by saprophytic fungi, in turn causing indirect injury to the plant. Sooty molds actually induce alterations of photosynthesis, disruption of metabolism, leaf curling and premature loss, together with lower production. Prolonged attacks and intense weakening by *C. pyri* may lead to plant death.

Fig. 1. Pear orchard with high plant density.

Unlike nymphs, adults of *C. pyri* (similarly to those of the congener species *C. pyricola*) are responsible for the transmission of the phytoplasma of "pear decline". The phytoplasma is acquired by the psylla upon feeding on an infected plant and transmitted to an healthy one by salivation during feeding on phloem (Carraro *et al*., 1998). The transmission mechanism is persistent-propagative because the phytoplasm reproduces in the insect body. The acquisition and inoculation of the pathogen requires for the vector insect to feed for 1-2 hours on phloem of infected plants. A latency period in the vector (about 1-2 weeks) follows the acquisition of phytoplasma in which the pathogen circulates and multiplies until it reaches the salivary glands. The first symptom of the disease appears during the summer-autumn period, when the leaves exhibit a red-purple hue contrasting with the yellow-green hue of senescent leaves of healthy plants. The leaves also have a stiff lamina with the edges curled upside and the apex folded downwards, and they fall prematurely beginning from the apical ones. In the next spring the infected trees show smaller, light green leaves with upward edges ("transparent canopy"). In some cases a sudden wilting is observed: although still on shoots, leaves become brown and dry. The tree may die within few days or weeks. Some authors (Giunchedi & Refatti, 1997; Davies *et al*., 1998) showed that some plants undergo recovering during the winter dormancy because of degradation of aboveground phloem elements (Schaper & Seemüller, 1982). Moreover, the quinces *Cydonia oblonga* Mill used as rootstocks rarely allow the phyoplasma survival between vegetative cycles, unlike *P. communis* rootstocks which allow phytoplasma reproduction within roots in winter and in the next spring, and the following spreading of the pathogen in aboveground phloem elements. Therefore in pear trees grafted to quince rootstocks the phytoplasma population, in absence of reinfection from overwintering psylla adults, may progressively decrease, disappearing within some years.

Fig. 2. Damages to fruits due to sooty molds.

Fig. 3. Damages caused by phytoplasmas.

3. The key to improve *C. pyri* control is the detailed knowledge of its life cycle

In Europe *C. pyri* shows 4-5 generations per year and overwinters as adult in reproductive diapause. The adult winter forms appear at the beginning of September (Fig. 4) (Civolani & Pasqualini, 2003) and overwinter individually or in small groups sheltered in cracks of the

tree bark, at branch crossing or at the base of buds. As soon as the weather conditions improve, the winter forms leave their shelter and reach the new apical leaves. Here they puncture the buds with their stylets, sucking and excreting fecal droplets which accumulate near the proctiger. In winter the adult male produces active sperms stored in the spermatheca and is therefore ready to mate and inseminate the female. However, the eggs can be fertilized only when oocytes reach maturity (Bonnemaison *et al*., 1956). In January all females are mature, ready to mate and lay eggs. The main factor affecting the physiology of ovopository apparatus is temperature, which must be higher than 10° C for two consecutive days (thermal quiescence) (Nguyen, 1975).

Fig. 4. *C. pyri* adult winter form.

Fig. 5. *C. pyri* adult summer form.

From eggs laid in winter months, simultaneously to bud opening, the first instar nymphs emerge, infesting the new vegetation. Later, in April-May, the adult summer forms appear (Fig. 5) whose females lay a large number of eggs (Fig. 6) hatching in the second half of May. The first instar (Fig. 7) and late instar nymphs (Fig. 8) live on the shoots, excreting a large amount of honeydew responsible for heavy damages to the plant. The next generations overlap with all developmental stages until autumn. Aestivation is also observed during the summer months.

Fig. 6. *C. pyri* eggs.

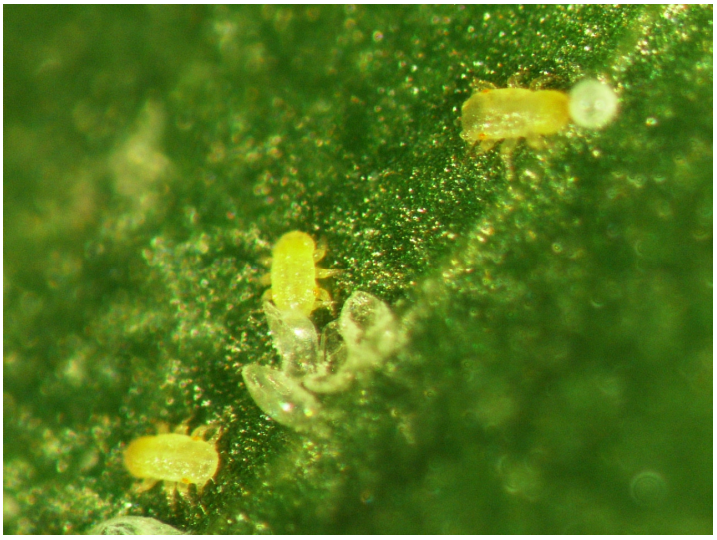

Fig. 7. Newly hatched *C. pyri* nymphs

Fig. 8. *C. pyri* late instar nymphs.

4. *C. pyri* Integrated pest management

In Europe this pest was considered of secondary importance until 1960, but its population rapidly increased simultaneously to heavy use of pesticides in pear orchards and intensive agriculture. The need to control pear infestations required the repeated use of different pesticides and several control strategies, but in most cases the efficiency of control progressively decreased, first because of the great adaptability and survival of the insect to the active ingredients, and second because of the negative effects on beneficial insects caused by excessive use of non-selective toxic ingredients. In this way the psylla population freely increased, leading to conditions in which the pest was hard or impossible to control.

Presently the defence against *C. pyri* is mainly based on integrated pest management (IPM), supported by natural control aimed to equilibrate the complex biological relationships of the field community. First of all, *C. pyri* control must follow IPM guidelines to optimize the activity of natural control agents and to reduce the chances of developing pesticide resistance by insect populations.

Among the basic strategies there are the "good agricultural practice" (GAP) techniques, such as reducing excessive plant growth and avoiding overstimulation by fertilizers or incorrect pruning. Also the general strategies to control other pest species may play a relevant role in psylla development and population increase. For example, the technique of mating disruption and the use of granulosis virus (CpGV) employed to control *Cydia pomonella* L. (Lepidoptera Tortricidae), favours the activity of entomophagous fauna by reducing the impact of synthetic products on the orchard, and may also help to reduce the treatments against *C. pyri*. However, in the last decade the chemical control has been the key defence strategy against the pear psylla in intensive pear orchards.

The main strategies of chemical control against *C. pyri* performed in the last 20 years in integrated and conventional farms are listed below. Each strategy has advantages and disadvantages, therefore its efficiency depends both on the active ingredients employed and the weather conditions at the time of treatment.

4.1 Autumn treatment

This treatment, performed at leaf fall, requires pesticides active against adult winter forms such as those belonging to the pyrethroid family (the same principles are also used in late winter against the same generation). These pesticides are completely non-selective, therefore dangerous for the beneficial insects: for this reason the treatment must be performed not too early (in October) when most individuals of *Anthocoris nemoralis* F. (Hemiptera Anthocoridae) are still active on the pear trees, but only at complete leaf fall (late November or early December), when *A. nemoralis* populations have already found shelter in bark crevices while *C. pyri* adult winter forms are still active on plants (Fig. 4) (Civolani, 2000; Civolani & Pasqualini, 2003).

Synthetic pyrethroids exhibit a very high abatement-contacticide action on psylla wintering adults. Table 1 shows the results of tests with the active ingredient ciflutrin: the abatement activity on adults is such that no egg deposition occurs in the next spring. Notwithstanding the good results that may be obtained by pyrethroid treatment in autumn (and also in late winter, as shown below) these strategies, common in France on *C. pyri* and in North America on *C. pyricola*, in the Italian pear growing regions are discouraged: here the pest population, after an initial sharp decline, soon recovers and increases again in May, reaching the economic threshold for spring-summer treatments. This event could be explained by the faster recovery of the pest in spring because the natural control by its predator *A. nemoralis* is limited. Moreover, considering only the pesticide efficiency, the results are strictly dependent on seasonal conditions. Indeed, if an early frost occurs at the beginning of autumn, most psylla adults take shelter early and survive to the late autumn treatments. Given the need to preserve auxiliary insect populations, an untimely autumn treatment with broad spectrum pyrethroids could be very dangerous for them and therefore is strongly discouraged in relevant pear growing areas such as Emilia-Romagna (Northern Italy).

Experimental tests have been performed to evaluate other active principles such as mineral oils and neonicotinoids. According to tests performed in 2001-2002 in the province of Ferrara (Emilia-Romagna), good results were obtained with mineral oil alone or associated to imidacloprid (Table 1) (Civolani, 2000), although for auxiliary insect preservation the use of neonicotinoids is not suggested against *C. pyri* in European pear growing areas, unlike North America where is found in field control guidelines against *C. pyricola*.

	Treatment date	Eggs per 100 bubs
untreated control	-	170
mineral oil	29 November 1999	30
mineral oil + imidacloprid	29 November 1999	12
ciflutrin	29 November 1999	0
mineral oil	3 March 2000	53
mineral oil + imidacloprid	3 March 2000	65
ciflutrin	3 March 2000	69

Table 1. Results of a field test with a pyrethroid and mineral oil with or without a neonicotinoid (Civolani, 2000).

4.2 Late winter treatment

This treatment is presently based on broad spectrum pyrethroids whose purpose is to break down the population of females emerging from winter shelters and about to lay eggs.

Formerly the highly toxic 4,6-dinitro-*o*-cresol (DNOC) was used, until finally banned in 1999. With a biological activity extended up to 15 days, DNOC was characterized by a strong contact activity especially towards eggs newly laid or about to be laid (Barbieri et al., 1986; Pollini et al., 1992). On pear psylla, the pyrethroids are exclusively active on adults through contact associated to an anti-feeding effect. The pyrethroid treatment is generally performed at pear bud swelling stage or at the latest when they break. The eggs are laid by *C. pyri* at the end of the thermal quiescent period, corresponding to a maximum temperature above 10° C for two consecutive days. The late winter strategy considerably lowers the amount of initial psylla population on pear trees, therefore the first generation is extremely reduced.

Concerning the pesticide activity, the best results of this strategy are obtained after a mild winter, because in these conditions almost all adults leave their shelters at the time of treatment. On the contrary, frost waves at the end of winter interrupt and delay the emergence of adults, reducing the efficiency of the treatment.

The control strategy based on late winter treatments with pyrethroids was recently disputed first because of the toxicity of the active ingredients involved, but mostly because the sharp reduction of the psylla first generation could starve the anthocorids, interfering with their settlement during early plant growth in spring. To avoid the side effects of pyrethroids, in the last ten years alternative solutions to synthetic pesticides have been repeatedly tested against the overwintering generations, and among them kaolin and some oily compounds.

Kaolin, a white, non-abrasive, fine-grained allumosilicate mineral that is purified and sized so that it can be easily dispersed in water, creates a mineral barrier on plants that prevents oviposition and insect feeding (Fig. 9 and Fig. 10) (Puterka et al., 2000). The efficiency of kaolin is shown by the results obtained in two experimental trials in 2001 and 2002 (Pasqualini et al., 2003). These authors found that a double treatment with kaolin affected egg laying of overwintering *C. pyri* by hindering their anchorage on the leaf surface and inhibiting host-plant acceptance (Table 2). It was also found that the body and wings of some adults became soiled, making insects less mobile and preventing them from reaching the laying site (host location) on plants. Indeed, the kaolin-treated plants were almost completely free of nymphs (Table 2). Due to the high mobility of *C. pyri* adults, the effects of kaolin treatment on the summer population were not assessed in this small plot trial. A larger plot trial was therefore performed in 2004 and again kaolin showed a very high control efficiency (Daniel et al., 2006). At the end of June 2004 some *C. pyri* individuals were observed in the kaolin treated plot, but the population density tended to be lower than that in the plot treated by a standard spirodiclofen strategy. In kaolin treated plants *C. pyri* was kept under the economic threshold until harvest (Daniel et al., 2006), therefore it could be an interesting alternative control strategy for this pest in organic and IPM orchards.

Some oily compounds could also be used in this period to interfere with egg deposition by *C. pyri* adults. It has been known from a long time that mineral oils and oily compounds could have negative effects on psylla egg deposition (Zwick & Westigard, 1978). A Turkish researcher (Erler, 2004) tested four types of oils, namely cotton seed oil, neem oil, fish-liver oil and summer oil, observing a delay in egg laying of about four weeks for fish-liver oil and summer oil, but of only one or two weeks for cotton seed oil and neem oil, probably depending on the stability of the oily material in open field. Other field trials aimed to interfere with egg laying by *C. pyri* adult winter forms were performed in 2001 and 2002 in Italy with pure mineral oil ("dormant oil"), obtaining a good reduction of the number of eggs laid (Pasqualini et al., 2003) (Table 2).

Fig. 9. Pear trees treated with kaolin in late winter.

Fig. 10. *C. pyri* adult with body soiled by kaolin.

	Treatment date	Eggs per 100 bubs	Nimphs per 100 flowers
First trial 2001			
Untreated control	-	136.75	6
kaolin	18 February and 10 March	1	0.25
mineral oil (dormant oil)	18 February.	30	2
Second trial 2002			
Untreated control	-	77.75	7.5
kaolin	11 and 19 February	0	0
mineral oil	11 February	12	3.5

Table 2. Results of two late winter field trials with kaolin and mineral oil (Pasqualini et al., 2003).

4.3 Spring-summer treatments

The treatments against summer generations can be performed towards eggs or nymphs. In the first case chitin inhibitors such as hexaflumuron (banned in 2004), triflumuron (banned in 2009), diflubenzuron and teflubenzuron have been employed. These active ingredients are used against second generation eggs, usually laid in the first decade of May. The treatment is usually performed against *C. pomonella* but shows a secondary effect on psylla. The chitin inhibitors provide the best results on *C. pyri* when they are applied on newly laid eggs (white eggs) or on eggs laid in a short time after the treatment. However, no activity is clearly exerted on eggs laid on the new shoots unreached by the treatment. Some authors observed that chitin-inhibitors show control effects similar to those of specific psyllicides described below: this could be explained by the absence of side effects on *C. pyri* natural predator, *A. nemoralis* (Souliotis & Moschos, 2008). However, the most relevant treatment employed against *C. pyri* in past and present times is the one against juvenile stages. This control strategy is base on specific synthetic active ingredients which are often acaricides, such as amitraz (commercially released in 1975 and banned in 2005), abamectin (commercially released in Italy in 1996) and spirodiclofen (commercially released in Italy in 2007), although in the past generic organophosphorates have been used such as monocrotofos and azinphos methyl.

Abamectin is presently the basic pesticide employed against *C. pyri*. Is should be briefly recalled that abamectin belongs to the chemical family of avermectins, compounds produced by the soil bacterium *Streptomyces avermitilis* (Lasota & Dybas, 1991). The activity of abamectin is mainly directed against young nymphs and secondarily against adults. The best results are therefore obtained when yellow eggs are mostly present and when the hatching peak, that could interfere with the pesticide activity because of honeydew, has not yet achieved (Pasqualini & Civolani, 2006). The product is not systemic but translaminary: the addition of mineral oil improves its penetration and after 24 hours no traces of the compound are found on leaf surface. One treatment timely performed against the second generation often represents the final solution, considering the high activity of the principle in comparison to amitraz (Table 3).

Spirodiclofen, commercially available since 2007, is the first member of a new chemical family, that of the tetronic acids (Nauen et al., 2000; Nauen, 2005), characterized by a new and original

mechanism of action which interferes with biosynthesis of lipids in the target arthropods. The original mechanism of action of spirodiclofen plays a key practical role by allowing successful rotation strategies with abamectin to limit the risks of occurrence of resistance in *C. pyri* control. The best activity of spirodiclofen occurs when it is targeted on yellow eggs some days before the hatching of first instar nymphs: the active ingredient shows instead a decreasing activity with the advancing of the psylla developmental stages (Table 4). At the stage of yellow eggs the activity of spirodiclofen is improved by addition of mineral oil (Table 4) and the use of spirodiclofen followed by treatment with mineral oil (1000 ml/hl) may represent a good alternative to the treatment with abamectin in presence of high and prolonged infestations. In conditions of low-medium pressure by *C. pyri*, one treatment with spirodiclofen may be enough: the few individuals escaping the treatment may be easily captured by anthocorids, given the good selectivity of the product towards these valuable auxiliary insects (Pasqualini & Civolani, 2007). However, the efficiency of spirodiclofen is often lower than that of abamectin (Table 4) (Pasqualini & Civolani 2007; Boselli & Cristiani 2008; Marčić et al., 2009).

Other active ingredients have been employed on both *C. pyri* in Europe and *C. pyricola* in North America. For example, in *C. pyricola* the differences of abamectin efficiency observed in the field suggested to employ and recommend neonicotinoids in pear IPM programs, among which imidacloprid, introduced in 1995, thiametoxan in 2001, acetamiprid in 2002 and thiacloprid in 2004. Besides *C. pyricola*, the last ingredient is used mainly for codling moth *C. pomonella*.

Presently a new active ingredient, spirotetramat, a lipid biosynthesis inhibitor similar to the tetronic acid derivate spirodiclofen, is under investigation in Europe (Nauen et al., 2008) but already commercially available in North America. Due to its mode of action spirotetramat is especially effective against juvenile stages of sucking pests, psyllid included. In the case of female adults the compound significantly reduces fertility and consequently insect populations. Spirotetramat also exhibits unique translocation properties: after foliar uptake the insecticidal activity is translocated within the entire vascular system. This property allows the protection of new shoots or leaves appearing after foliar application (Nauen et al., 2008): given the high efficiency on *C. pyri* (unpublished data) this active principle could represent a future valuable alternative to abamectin in order to manage the risks of occurrence of resistance in *C. pyri* control.

It is also possible to control nymphs by simply washing the trees with high amounts of water to which insecticidal soaps (fatty acids salts) are added to remove the honeydew (Briolini et al., 1989). Recently some other products have been used, similar to liquid glue and able to trap by a physical mechanism small and scarcely active insects such as almost all juvenile instars of *C. pyri.* These products are synthetic sugar esters (sucrose octanoate) and represent a relatively new class of insecticidal compounds that are produced by the reaction of sugars with fatty acids. (Puterka et al., 2003).

After discussing the active ingredients that could be used against *C. pyri* and the different strategies that could be employed, once again it must be emphasized that an efficient control of *C. pyri* infestations could be obtained by an integrated pest management of the pear orchard which allows a balanced growth of plants and simultaneously favours the growth of populations of natural psylla antagonists.

During the spring growth period a key point is to protect the useful psylla predators, first of all the most important one, *A. nemoralis*. The populations of this anthocorid are low in early spring but increase in the second half of June, insuring the protection of pear trees until harvesting and providing the most relevant contribution against *C. pyri*.

Another aspect that should not be overlooked in *C. pyri* control is the relevant effect of weather conditions on pest populations. A late winter season with mild temperatures favours an early and fast emergence of adults from their winter shelters and a regular egg laying, while the late winter frosts interrupt the adult emergence and egg laying, producing a gradual development of first and second pest generation which interferes with the precise timing of treatments. Cold and rainy periods during blossoming and petal fall interfere with nymph spreading on plants: in this case the nymphs often crowded in the flower calyx, sometimes causing with their feeding activity russet blotches or young fruit drop. On the contrary, high summer temperatures tend to block psylla development because of high egg mortality and slowing of juvenile growth for a long period.

	Treatment date	Nymphs per shoot
First trial (2000, on the variety "Conference")		
untreated control	-	16.68
abamectin	6 May	0.15
amitraz	10 May	1.08
Second trial (2000, on the variety "William")		
untreated control	-	46.88
abamectin	6 May	2.23
amitraz	10 May	8.18
Third trial (2004, on the variety "Conference")		
untreated control	-	8.29
abamectin	6 May	0.98
amitraz	10 May	1.39

Table 3. Results of three field trials with abamectin and amitraz on two pear varieties (Pasqualini & Civolani, 2006).

5. Evolution of resistance of *C. pyri* to pesticides

As for all phytophagous pests, also for *C. pyri* the repeated use of chemical active ingredients causes the development of resistance. However, in Europe there are less resistance cases documented for *C. pyri* in comparison to those known since 1960 for *C. pyricola* in North America (Harries & Burts, 1965). Among the *C. pyri* resistance events in Europe the best known involve organophosphorates, pyrethroids and chitin inhibitors: in all documented cases a sharp decrease in pesticide activity was observed even after a few years of use. The active ingredient monocrotofos represents the best known case (Berrada *et al.*, 1995). The selection induced by this pesticide around the end of 1980 on some *C. pyri* populations near Toulouse (France) caused an increased resistance up to 140 fold in comparison to the susceptible strain in 30 generations, as shown by laboratory tests. Further tests showed that the mechanisms involved in the onset of resistance to this active

	Treatment date	Nymphs per shoot
First trial (2005, on the variety "Conference")		
untreated control	-	83.25
spirodiclofen	19 May (yellow eggs)	36.25
spirodiclofen + mineral oil	19 May (yellow eggs)	15.50
abamectin + mineral oil	19 May (yellow eggs)	0.75
Second trial (2002, on the variety "Abbé Fétel")		
untreated control	-	25
spirodiclofen	30 April (white eggs)	6
spirodiclofen	14 May (yellow eggs)	11
spirodiclofen	17 May (first hatching)	19
spirodiclofen	22 May (20-30 % hatching)	18
amitraz	14 May (yellow eggs)	2
Third trial (2007, on the variety "Beurré Bosc")		
untreated control	-	66.5
spirodiclofen	27 April	1.5
spirodiclofen and abamectin	27 April and 9 May	0
spirodiclofen and mineral oil	27 April and 9 May	0.6
abamectin	27 April	0.5
Abamectin and spirodiclofen	27 April and 9 May	0

Table 4. Results of three field trials with spirodiclofen on three pear tree varieties (Pasqualini & Civolani, 2007; Boselli & Cristiani, 2008).

ingredient were the enhanced activity of cytochrome P450 monooxygenase (MFO) and also the changes in acetyl cholinesterase, since the susceptibility to the pesticide could not be fully recovered by pretreating *in vivo* the adults with piperonylbutoxide (PBO), a specific inhibitor of MFO (Berrada *et al*., 1994).

In 1994 in the Avignon region (France) the survey for *C. pyri* resistance was extended to 16 active ingredients belonging to five pesticide families, by topical laboratory tests on adults (Fig. 11). The tests showed that the resistance rates (RR) were extremely low for the family of carbamates (one- to 2.4-fold), relatively low for the family of pyrethroids (4.7- to 6.2-fold). For the family of organophosphorates insecticides, the resistance rates among the active ingredients were very different: lower than one (0.2-fold) for parathion-methyl, low for mevinphos and malathion (3.5- and 2.5-fold, respectively), and higher for chlorpyriphos-ethyl (10.2-fold), monocrotophos (26-fold), azinphos-methyl (62.2-fold) and phosmet (179.7-fold). In the same Avignon area, tests on resistance selection were performed in laboratory with the organophosphorate azinphos-methyl, which was frequently used in high amounts against *C. pomonella* and could indirectly cause selection also in *C. pyri*. The RR observed ranged from 10 to 40-fold in comparison to wild populations: these values were

considerably lower than those reached by monocrotofos (Bues *et al*., 2000). Further tests showed that the selection by azinphos-methyl produced a strong cross resistance with phosmet and monocrotofos (155 fold). By the same tests no cross resistance was shown for azinphos-methyl with amitraz, pyrethroids, carbamates and other organophosphorate pesticides such as chlorpyriphos and mevinphos (Bues *et al*., 2000). The same authors started genetic studies by crossing the resistant and susceptible strains and showed that the resistance was autosomically inherited and semi-dominant in expression (Bues *et al*., 2000). They also advanced by backcross the hypothesis that the resistance factor was monogenic. However, the result that the resistance to azinphos-methyl is semi-dominant is different from what previously obtained in Oregon by Van de Baan (1988) on pyrethroids: this author crossed two populations of *C. pyricola*, one susceptible and the other 240-fold resistant to the active ingredient fenvalerate (a pyrethroid), showing that the resistance was in this case semi-recessive (Van de Baan, 1988). Probably there are different mechanisms involved in the resistance to different pesticide families.

As mentioned before, the pyrethroids are another pesticide family largely employed in France against *C. pyri* and in North America against *C. pyricola* during leaf fall and late winter, before egg laying by overwintering adults. In laboratory topical tests on overwintering adults, some of these active ingredients showed very variable RR: for example, in tests performed in Avignon in 1994 the observed RR were 4 or 6-fold higher in comparison to the susceptible laboratory population (Buès *et al*., 1999), but further studies in 1996 in the same southern Rhone valley, on a field population collected in Pont Saint Esprit, showed that the RR was 42.9-fold higher (Buès *et al*., 1999). Later, the same authors confirmed similarly high resistance values to the active ingredient deltametrin in some Southern France populations in which the RR was 30-fold, always with the adult winter forms less susceptible in comparison to the same stage of the adult summer forms (Buès et al., 2003).

Concerning the mechanism of action producing resistance to pyrethroids, laboratory tests showed that the addition of PBO caused an recovery of pyrethroid efficiency almost complete: this shows that other mechanisms of induction of resistance could be secondarily involved.

Therefore the detoxifying enzyme MFO is involved in the mechanism of action of both organophosphorate and pyrethroid active ingredients.

Around 1995, in a region of West Switzerland a lower susceptibility was observed to teflubenzuron, active ingredient belonging to the family of chitin inhibitor (Schaub *et al*., 1996). More recently this resistance to teflubenzuron was also observed in the Czech Republic, according to tests performed in 2004 and 2005 (Kocourek & Stará, 2006). The mechanisms of resistance to teflubenzuron have not yet been completely investigated.

As previously mentioned, abamectin is the most efficient and most used active ingredient against *C. pyri*: it was used against this pest for the first time in 1996, with only one spring treatment and only in some orchards, also because amitraz was an alternative until 2005, the year in which the ingredient was banned. Probably after the ban on this active ingredient the number of treatments per orchard and the area of employment were increased: this trend was also observed in the fruit growing area of Lleida, Girona and Huesca in Spain (Miarnau et al., 2010), and Ferrara and Modena in Italy. The current high selection pressure with this active ingredient, repeatedly applied over both geographical areas could induce selection for resistance.

Following the sudden outbreak of *C. pyri* populations in some fruit growing area in Emilia-Romagna in 2005, abamectin tests for resistance were performed for the first time only on overwintering adults (Fig. 11), but no resistance was detected, although LC_{50} and LC_{90} values appeared related to the time of adult field collection (Civolani et al., 2007).
Besides adults (Table 5) (Fig. 11), in 2007 and 2008 the abamectin tests in Emilia-Romagna were extended also to eggs and nymphs (Fig. 12 and Fig. 13), the stage target in *C. pyri* field treatments (Civolani et al., 2010). On adults, the abamectin topical tests performed in autumn 2007 and 2008 did not show significant differences among all populations tested, but the LC_{50} values were apparently related to the adult collection dates, as previously reported (Civolani et al., 2010).

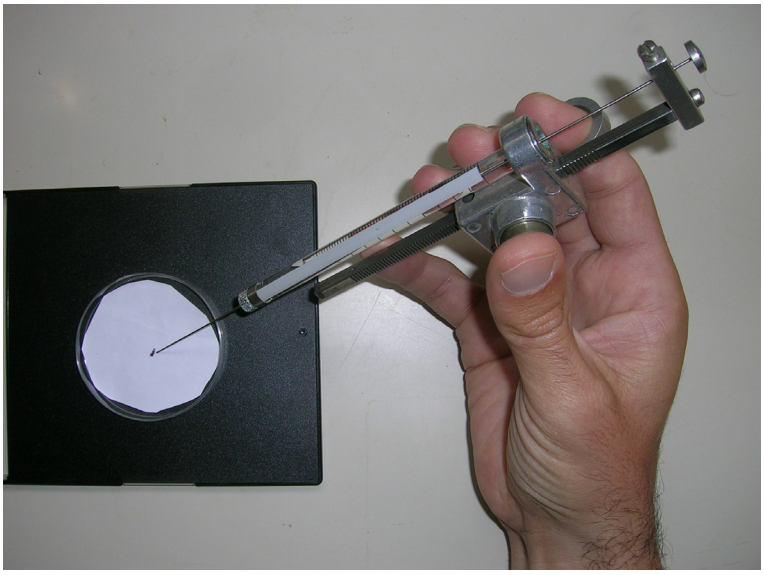

Fig. 11. Topical application of the insecticide solution on *C. pyri* adult winter form with hand-held manual micro-applicator.

The egg spray test (LC_{50} and LC_{90}) did not show relevant differences, although LC_{50} and LC_{90} values were always lower in the organic farm than in all others (Table 6). The results of leaf dip tests performed on young and old larvae were generally similar to the egg spray ones (Civolani et al., 2010).
Overall, the tests data indicate that no apparent resistance to abamectin has been developed up to now in *C. pyri* populations of Emilia-Romagna.
In 2009 and 2010 new cases of loss of efficiency of abamectin reappeared in some orchards in the province of Modena (Emilia-Romagna). In 2010 other tests were again performed on a *C. pyri* population which underwent 7 abamectin field treatment in 2009. However, in this case also the results of LC_{50} and LC_{90} did not show significant differences and the RR was just above 2 (unpublished data).
From 2004 to 2006 tests were carried out also in Spain to monitor the evolution of susceptibility to abamectin in orchards where the number of treatments changed from less than two until 2005 to an average of three after the ban on amitraz, with some orchards

undergoing 6-7 treatments. The tests were performed both on adults and nymphs and no evidence of a high RR was found (Miarnau et al., 2010). However, there are some Spanish populations of *C. pyri* that show low susceptibility in adults as well as in nymphs: these populations come from the fields with the highest number of abamectin treatments per year. As in Emilia-Romagna, these cases also indicate a high risk of selection for resistance to abamectin, especially if the number of treatments per year is high and there are no alternative products to use in a resistance management program.

Fig. 12. Cages for potted pear plants to keep adults of different population separated.

Fig. 13. Potted young pear plants on which psylla eggs were laid and then treated with abamectin.

Population (locality)	Field strategy	2007		2008	
		LC_{50}	RR	LC_{50}	RR
Buondì-Vezzani (Diamantina)	organic	3.13		5.27	
Minotti (S. Martino)	integrated	4.98	1.59	3.24	0.61
Scagnolaro (Francolino)	integrated	2.80	0.89	4.16	0.78
Bonora (Boara)	integrated	-	-	5.95	1.12
Celati (Francolino)	integrated	-	-	4.23	0.80
Marchetti (Diamantina)	conventional	-	-	5.31	1

Table 5. Response of *C. pyri* overwintering adult populations to topical applications of abamectin (2007 and 2008) in the province of Ferrara (Civolani et al., 2010). The values of LC_{50} are expressed in mg l^{-1} of abamectin.

Population (locality)	Field strategy	2007		2008	
		LC_{50}	RR	LC_{50}	RR
Buondì-Vezzani (Diamantina)	organic	0.20		0.15	
Minotti (S. Martino)	integrated	0.43	2.15	0.29	1.93
Scagnolaro (Francolino)	integrated	-	-	0.15	1
Bonora (Boara)	integrated	-	-	0.27	1.80
Celati (Francolino)	integrated	-	-	0.34	2.26
Marchetti (Diamantina)	conventional	-	-	0.44	2.93

Table 6. Response of *C. pyri* to egg spray applications (2007 and 2008) in the province of Ferrara (Civolani et al., 2010). The values of LC_{50} are expressed in mg l^{-1} of abamectin.

Spirodiclofen is a recently introduced active ingredient in *C. pyri* control. Different LC_{50} values were observed for spirodiclofen in tests again performed in Emilia-Romagna, regardless of its limited use as an alternative to abamectin. This active ingredient was tested on three populations of the province of Ferrara in 2006 and 2007 by laboratory assays on adults and eggs.

The topical tests on overwintering adults showed high susceptibility differences for this active ingredient between the adult population collected in the organic farm and those collected in the integrated (Minotti) and conventional (Marchetti) farms (Table 7).

Population (locality)	Field strategy	2006		2007	
		LC_{50}	RR	LC_{50}	RR
Buondì-Vezzani (Diamantina)	organic	19.09	-	89.01	-
Minotti (S. Martino)	integrated	2582.60	135.28	1931.70	21.,69
Marchetti (Diamantina)	conventional	25.93	1.358	943.72	10.60

Table 7. Results obtained in the topical test with spirodiclofen on *C. pyri* overwintering adults (2006 and 2007). The values of LC_{50} are expressed in mg l^{-1} of spirodiclofen.

The results obtained by the spray test on yellow eggs before hatching, using the field doses of spirodiclofen, show low activity on the population collected in the Minotti integrated farm (both on first and second psylla generation). The activity levels in the Minotti farm, expressed as percentage of nymph mortality, are very different from those detected in the other two farms. However, the values detected in the conventional Marchetti farm are unexpectedly similar to those obtained in the organic farm (Table 8): these results were surprising because spirodiclofen was never employed before in none of these farms (unpublished data).

Population (locality)	Field strategy	Nymph mortality % on eggs laid by overwintering adults	Nymph mortality % on eggs laid by adult summer forms
Buondì-Vezzani (Diamantina)	organic	100	94,91
Minotti (S. Martino)	integrated	30.62	77.95
Marchetti (Diamantina)	conventional	-	94.81

Table 8. Results obtained in the spray test with spirodiclofen on *C. pyri* eggs laid by overwintering and summer adults in 2007. The results are expressed as percentage of nymph mortality.

6. Natural and biological control of *C. pyri*

In open field and especially in the pear orchard the techniques of biological control are not common because the fruit growers have always aimed to favour and exploit the development of the wild auxiliary insects, thus performing strategies of natural control. *Antochoris nemoralis*, common in all Europe, is known as the main predatory species of pear psylla. This species overwinters as an adult (Fig. 14) and starts to lay eggs in spring, inserting them under the leaf epidermis. The development of the juvenile forms occurs in 5 stages. *A. nemoralis* preys on both eggs and nymphs of psylla and in Emilia-Romagna typically shows three generations. Although generally preferring psylla, this anthocorid may feed on other insects and on the pear trees its activity against aphids and the pear sawfly *Hoplocampa brevis* Klug (Hymenoptera Tenthredinae) is very interesting. Laboratory tests showed an average predation of about 300 psylla nymphs during the entire life of an adult, which lasts about 60 days. The presence of *A. nemoralis* in pear orchards mainly depends on the type of control strategy applied in the farm. As previously mentioned, by limiting the use of pesticides to the minimum required and preferring the selective ones (see next chapter), it is favoured the development of the wild *A. nemoralis* populations which become a relevant factor to control the pest. Indeed, a dynamic equilibrium develops between the predator and *C. pyri* populations, often leading to the solution of the problem without the need for specific chemical treatments, or with treatments only reduced to tree washing. The main problem of this strategy is nevertheless the slow initial development of the predator population, which must have the prey available (in this case *C. pyri*) to rapidly increase in number within the pear orchard. Therefore, in the initial part of the season (around May-June) it is necessary to tolerate some amounts of the pest in the orchard to obtain later a good number of predator anthocorids on the trees. In other words, this means

that when the presence of *C. pyri* rapidly increases in the third decade of May, usually we can expect a rapid increase of the predator population about two-three weeks later. This requires for the fruit grower to tolerate a relatively high presence of the pest and "resist" to the temptation to perform specific treatments. Since 1990, other field conditions added to these problems of the natural control, such as the increased chemical treatments against the codling moth, *C. pomonella*. These treatments caused a general weakening of the wild *A. nemoralis* populations, disrupting a very fluctuating natural equilibrium. Another problem recently emerged is that even in equilibrated pear orchards, not undergoing any heavy chemical treatment, for some reason the presence of predators remains low or undergoes high fluctuations over the years or even according to the seasons.

The previously mentioned limits of natural control led to artificially introduce anthocorids in the pear orchard, buying them from biofactories and thus performing a true biological control technique. The aim is to obtain a more numerous presence of the predator in the critical periods, anticipating the reproduction mechanism of the population which could occur naturally but with some delay. The introduction of predators is performed at the end of winter, between the end of March and the beginning of April. About 1000 adult individuals of *A. nemoralis* are placed per hectare of the pear orchard, in three consecutive weekly introductions. This biological control technique was common around 2000 in integrated pear orchards where the active ingredient amitraz was employed for the spring-summer control of *C. pyri*, then it was slowly neglected because of the limited results and the relatively high costs.

Fig. 14. Adult of *A. nemoralis.*

7. Selectivity of pesticides against *A. nemoralis*

In the last 20 years new families of pesticides have been developed with generally lower toxicity towards beneficial species in comparison to the previous ones. These pesticides are more suitable for the new techniques of integrated pest control (IPM) which preserve the contribution of beneficial insects to the natural control against pests, especially against *C. pyri*. Therefore it was necessary to perform tests on the new active ingredients, to verify their different toxicity degree and obtain the data required to improve their use.

Several studies have been recently performed to evaluate the selectivity of the new pesticides towards auxiliaries found in the pear orchards, first of all *A. nemoralis* (Fig. 14), the most relevant in the natural control against *C. pyri*. The active ingredients recently investigated include those directly employed against *C. pyri* (psyllicides) and also those largely employed on other key pests (non-psyllicides).

Concerning the psyllicides, in different toxicity tests performed in Emilia-Romagna since 1997 abamectin showed a medium degree of toxicity against the *A. nemoralis* population, mostly against first and second instar nymphs in comparison to adults. In some cases the mortality of nymphs reached 50% and, according to field data, the total population appears limited in comparison to untreated controls for about two weeks after the treatment. This result must nevertheless take into account the lower presence of prey as food for *A. nemoralis* (Pasqualini & Civolani 2007). Other products specific for *C. pyri*, such as amitraz (now banned), mineral oil, or insecticide soaps did not show relevant effects on the predator population (Civolani & Pasqualini, 1999; Pasqualini et al., 1999). Concerning spirodiclofen, the tests on toxicity towards *A. nemoralis* were performed again in Emilia-Romagna in years 2004-2006. The results show that the anthocorid populations undergoing treatment with spirodiclofen have similar development to the untreated ones, unlike abamectin for which the population first sharply decreases, then recovering according to the amount of prey available (Pasqualini & Civolani, 2007).

Concerning the non-psyllicides, those belonging to the chitin inhibitors, usually characterized by a long period of action, generally show a low toxicity on *A. nemoralis*, even if some of them, such as flufenoxuron, induce a heavy reduction of the anthocorid populations (Girolami et al., 2001, Pasqualini & Civolani 2002).

Among other non-psyllicides employed in pear orchards against the most relevant pests, there is spinosad, a pesticide of natural origin, whose activity derives from a toxin produced by *Saccharopolyspora spinosa* (Bacteria Actinomycetales), the indoxacarb belonging to the family of the oxadiazines, then methoxyfenozide and tebufenozide, synthetic molecules belonging to the family of moulting accelerator compounds (MAC), and the organophosphorates azinphos-methyl (banned in 2007), chlorpyrifos, chlorpyrifos-methyl and phosmet, all employed against relevant lepidopteran pests such as *C. pomonella*, *Cydia molesta* Busck and *Pandemis cerasana* Hübner. All these active ingredients have been more or less tested for toxicity against *A. nemoralis* and the results did not show relevant toxic effects on this predator (Civolani & Pasqualini, 1999).

As previously mentioned, the neonicotinoids are not employed in Italy and Europe as specific psyllicides, on the contrary of what happens in United States where they are employed as an alternative to abamectin against *C. pyricola*. However, a large amount of the above active ingredients are used against other pests, such as aphids and the pear sawfly *H. brevis*: the most relevant are imidacloprid, acetamiprid and tiametoxan, while against the codling moth *C. pomonella* the most frequently used is thiacloprid. All those active

ingredients have significant toxic effects on *A. nemoralis*, thus they must be employed only for very few treatments along the year.

8. Conclusions

Concerning the strategies and methods to control *Cacopsylla pyri* (Hemiptera Psyllidae), and also their effects on the beneficial insects and the development of resistance, during the past decade Italian and European populations of *C. pyri*, in significant decline, have been apparently less capable to induce damage: this could be due to the success of defence programs based on integrated pest management (IPM). For pear, the IPM involves pest population control and auxiliary insect protection, associated to availability of new chemical or microbiological agents specifically targeted to pear key pests (mainly *Cydia pomonella*).

As far as known about *C. pyri* infestation, the biological control alone is not successful in preventing damage, especially when caused by second generation nymphs that feed on shoots and leaves in late spring and summer; thus chemical pest control strategies are also employed. For example, in Northern Italy the most common defense strategy against *C. pyri* in pear orchards involves chemical treatments on second-generation eggs or nymphs. Traditional treatments with chitin inhibitors, mainly aimed against *C. pomonella*, have also some secondary effects on *C. pyri*.

Specific treatments against *C. pyri* nymphs involve amitraz (now banned), abamectin and spirodiclofen. During spring and summer the treatment with the last two active ingredients, in addition to non-chemical treatments such as tree washing, is usually successful in limiting the honeydew damages caused by *C. pyri*.

During autumn and winter, the *C. pyri* management strategies involve treatments with synthetic pyretroids after leaf fall, to limit adult overwintering population, or at the end of winter, when females are ready to lay eggs. However, these treatments during autumn and winter, common in France and North America, are rarely employed in Italy because of their high toxicity against populations of auxiliary insects (such as Anthocoridae) which could still be present in the pear orchards. In late winter it is also possible to perform a non-chemical treatment against *C. pyri* by distribution of an aqueous suspension of kaolin on trees, in order to obtain a physical barrier to egg laying.

Each one of the above strategies shows favorable and unfavorable aspects in terms of efficacy, side effects on beneficial insects, timing of application and environmental conditions.

As for all phytophagous pests, also for *C. pyri* the repeated use of chemical active ingredients causes the development of resistance. Indeed, several insecticides employed in the past to control the pear psylla showed a sharp decline in activity because of resistance development. After the sudden outbreak of *C. pyri* populations in some fruit growing area of Ferrara and Modena (Italy) and Lleida, Girona and Huesca (Spain), abamectin tests were performed on winter form adults and nymphs. The results did not show relevant resistance effects, although LC_{50} and LC_{90} values were always higher in populations where abamectin treatment was repeated several times in the year. Overall, the results indicate that no apparent resistance to abamectin has been yet developed in *C. pyri* populations of the most important European areas of pear growth: nevertheless, the pear orchards in which *C. pyri* outbreaks recently occurred are presently under close investigation and careful survey.

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10. References

Barbieri, R.; Becchi, R. & Pozza, M. (1986). Difesa fitosanitaria del pero, un quadriennio di prove e osservazioni anche con l'impiego di un piretrinoide in alternativa al D.N.O.C.. *Informatore Fitopatologico*, 36(12): 36-42

Berrada, S.; Fourner, D.; Cauny, A. & Nguyen T. X. (1994). Identifications of resistence meccanism in selected laboratory strain of *Cacopsylla pyri*, L. (Hom.: Psyllidae). *Pesticide Biochemistry and Physiology*, 48: 41-47

Berrada, S.; Nguyen, T. X.; Merzoug, D. & Fourner, D. (1995). Selection for monocrotophos resistance in Pear Psylla, *Cacopsylla pyri*, L. (Hom.: Psyllidae). *Journal of Applied Entomology*, 119: 507-510

Bonnemaison, L. & Missonnier, J., (1956). Le Psylle du poirier (*Psylla pyri* L.). Morfologie et biologie. Méthodes de lotte. *Annales des Epiphyties*, 7(2): 263-331

Boselli, M. & Cristiani, C. (2008). Diversificare le strategie di lotta contro la psilla del pero. *Terra e Vita*, 18: 64-67

Briolini, G.; Faccioli, G. & Pasqualini, E. (1989). A seven-year research on alternative methods to control Pear Psylla. *SROP/WPRS Bulletin*, 13(2): 89-92

Bues, R.; Boudinhon, L.; Toubon, J. F. & Faivre D'arcier, F. (1999). Geografic and seasonal variability of resistance to insecticides in *Cacopsylla pyri*, L. (Hom., Psyllidae). *Journal of Applied Entomology*, 123: 289-297

Bues, R.; Toubon, J. F. & Boudinhon, L. (2000). Genetic analysis of resistance to azinphosmethyl in the pear psylla *Cacopsylla pyri*. *Entomologia Experimentalis et Applicata*, 96: 159-166

Bues, R. ; Toubon, J. F. & Boudinhon, L. (2003). Resistance of pear psylla *Cacopsylla pyri* L.; Hom., Psyllidae to deltametrin and synergism with piperonil butoxide. *Journal of Applied Entomology*, 127: 305-312

Carraro, L.; Loi N.; Ermacora, N.; Gregoris, A.; Osler, R. & Hadidi, A. (1998). Transmission of pear decline by using naturally infected *Cacopsylla pyri* L.. *Acta Horticulturae*, 472: 665-668

Civolani, S. & Pasqualini, E. (1999). Tossicità nel breve periodo di diverse formulazioni di insetticidi su alcuni gruppi di entomofagi. *L'informatore Agrario*, 20: 87-90

Civolani, S. (2000). Elementi di studio sulla dinamica delle popolazioni svernanti di *Cacopsylla pyri* L. e del suo predatore *Anthocoris nemoralis* in relazione alla diffusione della moria del pero *Pyrus communis* L. in Emilia-Romagna. *Informatore Fitopatologico*, 50(11): 27-34

Civolani, S., Pasqualini, E., (2003). *Cacopsylla pyri* L. (Hom., Psyllidae) and its predators relationship in Italy's Emilia-Romagna region. *Journal of Applied Entomology*., 127: 214-220

Civolani, S.; Peretto, R.; Caroli, L.; Pasqualini, E.; Chicca, M. & Leis, M., (2007). Preliminary resistance screening on abamectin in pear psylla (Hemiptera: Psyllidae) in Northern Italy. *Journal of Economic Entomology*, 100(5): 1637-1641

Civolani, S.; Cassanelli, S.; Rivi, M.; Manicardi, G. C.; Peretto, R.; Chicca, M.; Pasqualini, E. & Leis, M. (2010). Survey of susceptibility to abamectin of pear psylla *Cacopsylla pyri* L. (Hemiptera: Psyllidae) in northern Italy. *Journal of Economic Entomology*, 103(3): 816-822

Daniel, C. & Wyss, E., (2006). Pre-flowering kaolin treatments against the European pear sucker, *Cacopsylla pyri* (L.). *Deutsche Gesellschaft für allgemeine und angewandte Entomologie*, 15: 263-268

Davies, D. L.; Clark, M. F.; Adams, A. N. & Hadidi, A. (1998). The epidemiology of pear decline in the UK. *Acta Horticulturae*, 472: 669-672

Erler, E. (2004). Oviposition deterrency and deterrent stability of some oily substances against the Pear Psylla *Cacopsylla pyri*. *Phytoparasitica*, 32(5): 479-485

Girolami, V.; Berti, M. & Coiutti, C. (2001). Tossicità di nuovi insetticidi IGR su antocoridi predatori della psilla *L'Informatore Agrario*, 30: 63-66

Giunchedi, L. & Refatti, E. (1997). Il problema della moria del pero. *Frutticoltura*, 10: 59-61

Kocourek, F. & Stará, J. (2006). Managment and control of insecticide resistant pear psylla (*Cacopsylla pyri*). *Journal of Fruit and Ornamental Plant Research*, 14 (3): 167-174

Harries, F. H. & Burts, E. C. (1965). Insecticide resistance in the pear Psylla. *Journal of Economic Entomology*, 58 (1): 172-173

Lasota, J. A. & Dybas, R. A. (1991). Avermectins, a novel class of compounds: Implications for use in arthropod pest control. *Annual Review of Entomology*, 36: 91-117

Marčić, D.; Perić, P.; Prijović, M. & Ogurlić, I. (2009). Field and greenhouse evaluation of rapeseed spray oil against spider mites, green peach aphid and pear psylla in Serbia. *Bulletin of Insectology*, 62(2): 159-167

Miarnau, X.; Artigues, M. & Sarasua, M. J. (2010). Susceptibility to abamectin of pear psylla, *Cacopsylla pyri* (L.) (Hemiptera: Psyllidae) in pear orchards of North-East Spain. *IOBC/wprs Bulletin* 54: 593

Nauen, R.; Stumpf, N. & Elbert, A. (2000). Efficacy of BAJ 2740, a new acaricidal tetronic acid derivative, against tetranychid spider mite species resistant to conventional acaricides. In: *Proceedings of the Brighton Crop Protection Conference on Pests and Diseases*, 453-458

Nauen, R. (2005). Spirodiclofen : Mode of action and resistance risk assesment in Tetranychid pest mites. *Journal of Pesticide Science*, 30 : 272-274

Nauen, R.; Reckmann, U.; Thomzik, J. & Thielert, W. (2008). Biological profile of spirotetramat (Movento®)- a new two-way systemic (ambimobile) insecticide against sucking pest species. *Bayer CropScience Journal*, 61(2): 245-278

Nguyen, T. X. (1975). Évolution de la diapause ovarienne de *Psylla pyri* (Homoptera Psyllidae) dans les conditions naturelles de la région Toulousaine. *Bulletin de la Societe Zoologique de France*, 100(2): 241-245

Pasqualini, E.; Civolani, S.; Vergnani, S.; Cavazza, C. & Ardizzoni, M. (1999). Selettività di alcuni insetticidi su *Anthocoris nemoralis* F. (Heteroptera Anthocoridae). *L'Informatore Agrario*, 46: 71-74

Pasqualini, E. & Civolani, S. (2002). Mimic-Confirm (a. i. Tebufenozide): a tool for a soft and ecologically sound pest control in pear orchards. *IOBC/wprs Bulletin,* Vol. 25 (11): 97-106

Pasqualini, E.; Civolani, S. & Corelli Grappadelli, L. (2003). Particle film technology: approach for a biorational control of *Cacopsylla pyri* (Rhynchota: Psyllidae) in Northern Italy. *Bulletin of Insectology*, 55: 39-42

Pasqualini, E. & Civolani, S. (2006). Difesa della psilla del pero con abamectina. *L'Informatore Agrario*, 12: 50-54.

Pasqualini, E. & Civolani, S. (2007). Spirodiclofen, nuovo insetticida efficace contro la psilla del pero. *L'Informatore Agrario*, 11: 89 93

Pollini, A.; Bariselli, M. & Boselli, M. (1992). La difesa del pero dalla Psilla. *Informatore fitopatologico*, 5: 19-24

Puterka, G.; Glenn, D. M.; Sekutowski, D. G.; Unruh, T. R. & Jones S.K. (2000). Progress toward liquid formulations of particle films for insect and disease control in pear. *Environmental Entomology*, 29: 329-339

Puterka, G.; Farone, W,; Palmer, T. & Barrington, A. (2003). Structure-function relationships affecting the insecticidal and miticidal activity of sugar esters. *Journal of Economic Entomology*, 96: 636-644

Scaub, L.; Bloesch, B.; Bencheikh, M. & Pigeaud, A. (1996). Spatial distribution of teflubenzuron resistance by pear psylla in Western Switzerland. *OILB/SROP Bulletin,* 19 (4): 311-314

Schaper, U. & Seemmuller, E. (1982). Condition of the phloem and the persistence of mycoplasma-like organism associated with apple proliferation and pear decline. *Phytopathology*, 72: 736-742

Souliotis, C. & Moschos, T. (2008). Effectiveness of some pesticides against *Cacopsylla pyri* and impact on its predator *Anthocoris nemoralis* in pear-orchards. *Bulletin of Insectology*, 61: 25-30

Van De Baan H. E., (1988). Factors influencing pesticide resistance in *Psylla pyricola* Foerster and susceptibility in its Mirids predator, *Deraeocoris brevis* Knight. *Thesis, Oregon State University*

Zwick, R. W. & Westigard, P. H., (1978). Prebloom petroleum oil applications for delaying pear psylla (Homptera: Psyllidae) oviposition. *Canandian Entomologist*, 110: 225-236

18

Effects of Kaolin Particle Film and Imidacloprid on Glassy-Winged Sharpshooter (*Homalodisca vitripennis*) (Hemiptera: Cicadellidae) Populations and the Prevention of Spread of *Xylella fastidiosa* in Grape

K.M. Tubajika[1], G.J. Puterka[2], N.C. Toscano[3], J. Chen[4] and E.L. Civerolo[4]
[1]*United States Department of Agriculture (USDA), Animal and Plant Inspection Service (APHIS), Plant Protection and Quarantine (PPQ), Center for Plant Health Science and Technology (CPHST), Raleigh, North Carolina,*
[2]*United States Department of Agriculture (USDA), Agricultural Research Service (ARS), Stillwater, Oklahoma;*
[3]*Department of Entomology, University of California, Riverside, California,*
[4]*United States Department of Agriculture (USDA), Agricultural Research Service (ARS), San Joaquin Valley Agricultural Sciences Center (SJVSC), Parlier, California, USA*

1. Introduction

The glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar), (Hemiptera: Cicadellidae), is a major pest of important agronomic, horticultural, landscape, ornamental crops and native trees in California (Blua et al., 1999; Purcell et al., 1999; Purcell & Saunders, 1999). This insect is an invasive species in California that was first detected in the state in 1989 (Sorensen & Gill, 1996). This sharpshooter is a key vector of *Xylella fastidiosa* and has changed the epidemiology of *X. fastidiosa* (*Xf*) diseases affecting important agronomic and horticultural crops as well as landscape, ornamental and native trees in California based on the infection of these crops by the bacteria (Blua et al., 1999; Purcell et al., 1999; Purcell & Saunders, 1999) (Fig. 1). It is not clear if management strategies for Pierce's Disease (PD) developed in GWSS-free regions of California are suitable to manage the disease in vineyards where GWSS has become established.

Data from earlier studies suggest that GWSS has continued to increase in number (population density) and geographical range in California (Purcell & Saunders, 1999; Tubajika et al., 2004). GWSS populations are widely distributed over a large number of hosts including perennial agronomic crops, ornamental plantings, and weedy plant species (Hill & Purcell, 1995; Purcell et al., 1999; Raju et al., 1980). Previous studies by Blua et al. (1999) and Perring et al. (2001) showed that GWSS populations utilize citrus plants as their primary over-wintering host (other plants are available) when grapes, stone fruits, ornamental hosts,

and weedy species are dormant during the winter. This vector may have a great impact on grape-growing areas in the Coachella, Temecula and the lower San Joaquin Valley due to numerous citrus orchards that support overwintering populations. Effective insect and disease management strategies are dependent upon knowledge of inoculum sources, and biology and the ecology of insect vectors and their natural enemies (Blua et al., 1999; Hill & Purcell, 1995; Tubajika et al., 2004). The GWSS transmits *Xf* to grape (Fig. 1), as well as to oleander (Costa et al., 2000) with the transmission efficiency greater than that of other vectors such as the green sharpshooter (GSS) [*Draeculacephala mineroa* Ball] and red-headed sharpshooter (RHSS) [*Carneocephala fulgida* Nottingham], RHSS) (Purcell & Saunders, 1999), but much less than that of the blue-green sharpshooter (BGSS) [*Graphocephala atropunctata* (Signoret] (Purcell & Saunders, 1999).

(a) GWSS adult

(b) GWSS nymph

(c) GWSS egg

(d) PD symptoms in grape

Fig. 1. Glassy-winged sharpshooter (GWSS) adult (a), nymph (b) (photos by B. Stone-Smith), and egg (c) found in grape in Bakersfield, CA. The GWSS transmits the bacterium *Xylella fastidiosa*, which causes Pierce's disease (PD) (d) in grape.

Imidacloprid, (Admire, Bayer Corp., Kansas City, MO, USA), is an neonicotinoid insecticide which interrupts the binding of nicotinergic acetylcholine in post-synaptic receptors of the insect (Hemingway & Ranson, 2005; Romoser & Stoffolano, 1998). In Georgia, treatment of grapevines with Admire slowed the rate of PD spread, but ultimately extended vineyard life by only a year when infestations were severe (Krewer et al., 2002). Imidacloprid is the most widely used neonicotinoid for the prevention of infestation of grapevines by GWSS in California. This product may be useful in reducing *Xf* transmission by the GWSS and in

slowing the development of PD in grape. Contact insecticides offer short-term protection against GWSS infestations because of the continued movement of sharpshooter adults from citrus and non-treated hosts/areas which re-infest the grapevines. In a study on the transmission of *Xf* to grapevines by *H. vitripennis* (syn. *coagulata*), reported that *H. vitripenns* (syn. *coagulata*) transmitted *Xf* to grapevines in a persistent manner(Almeida & Purcell, 2003). This research also showed that the nymphal lost infectivity during molting, but there was no evidence of a latent period (delay after acquisition) of the pathogen by adult populations.

Likewise, kaolin (Surround®, NovaSource Tessenderlo Kerley, Inc., Phoenix, AZ.) is a potential alternative pest management product with improved safety to pesticide handlers and reduced environmental impact (Glenn et al., 1999)). It protects plants from insect feeding and oviposition by coating the plant surfaces with a protective mineral barrier (aka particle film) (Glenn et al., 1999; Puterka et al., 2000). Kaolin has been shown to suppress pear psylla, *Cacopsylla pyricola* (Forster); the spirea aphid, *Aphis spiraecola* Pagenstecher; the two-spotted spider mite, *Tetranychus urticae* Koch; and the potato leafhopper, *Empasca fabae* (Harris) in previous research (Glenn et al., 1999) (Fig. 2). The mechanism involved in reduction of pest density is reported to be reduced oviposition and feeding on treated plants (Glenn et al., 1999). Puterka et al. (2000) found Surround® reduced *C. pyricola* populations and controlled Fabraea leaf spot, fungal disease caused by *Fabraea maculata* Atk. Moreover, Glenn et al., (1999) reported that kaolin films contributed to control of fungal and bacterial plant pathogens by preventing the formation of a liquid film on the surface of pear leaves. In this chapter, the effects of kaolin particle film and imidacloprid on *H. vitripennis* (syn. *coagulata*) population levels and the prevention and spread of *Xf* in grape are described.

(a) Field study using kaolin (b) Close-up of kaolin-treated grape leaves

Fig. 2. Field study showing the application of kaolin (a) and a close-up of kaolin-treated grape used to control Glassy-winged sharpshooter in Kern County, Bakersfield in California.

2. Impacts of kaolin and imidacloprid on *Homalodisca vitripennis*

The use of insecticides is common for controlling pest populations (Hemingway & Ranson, 2005). However, the development of resistance and cross-resistance among widely used and newly applied systemic insecticides can affect the development of new strategies in integrated pest management. The GWSS has become a significant problem to California

agriculture because it feeds readily on grape and, in doing so, transmits *Xf,* the causal agent of Pierce's Disease (PD) in grape (Fig.1d). Prior to the appearance of GWSS, California grape growers were able to manage PD in grape that is vectored by a number of other indigenous sharpshooters (Anon, 1992). Unfortunately, the GWSS more adapted to the citrus/grape agroecosystem than other sharpshooters, thus, making it a serious vector of PD that now threatens the grape industry in California (Purcell & Saunders, 1999). Although contact insecticides offer short-term protection against infestations of GWSS, the continued movement of GWSS adults from citrus and uninfested areas often re-infest grapes. Clearly, there is a need to investigate other approaches or technologies that could repel GWSS infestations or prevent them from feeding on grape vines and transmitting PD (Puterka et al., 2003).

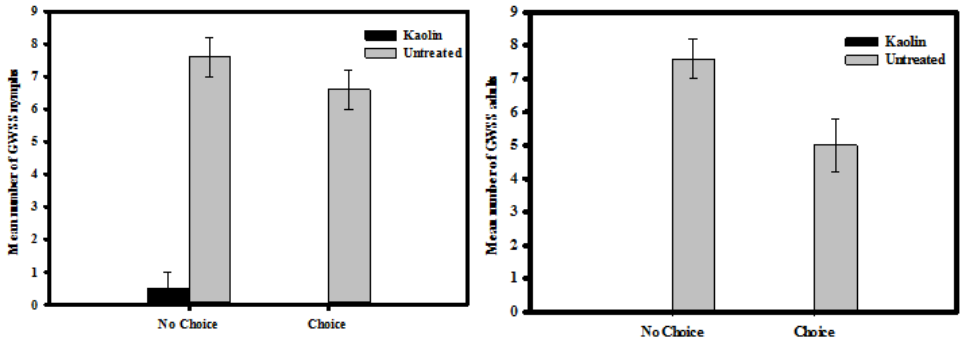
In an effort to assess the efficacy of control using kaolin, experimental potted lemon trees in field cages at the Bakersfield location were used to test the efficacy kaolin on GWSS infestation and survival under no-choice conditions. 'Eureka' lemon trees were either treated with 6% kaolin (60 grams kaolin per liter of water) or left untreated as a control. Trees were sprayed with kaolin by a 4-liter hand-pump sprayer until all of the foliage was wetted and then allowed to dry. Either a treated or untreated tree was placed in screen-covered cages measuring 1 m^2 by 2 m high, and either 50 GWSS adults (Fig. 1a) or 10 instar nymphs (third to fourth stage) (Fig. 1b) were placed at the bases of the trees to disperse. Numbers of GWSS on trees and within the cages were recorded daily for four days to determine infestation rates and survival. GWSS numbers were converted to percentages and analyzed using analysis of variance (ANOVA) (SAS Institute Inc., Cary, NC).

2.1 Survival effect of kaolin and imidacloprid on GWSS in citrus

Experimental results showed that treatment of grapes with kaolin significantly affected ($P < 0.01$) the number of nymph and adult populations. GWSS nymphs and adults were greatly repelled by kaolin application in treatments where choice and no-choice experiments were conducted (Fig.3ab). Very few nymphs or no adults were able to colonize lemon treated with kaolin in experiments where either GWSS nymphs or adults were given no choice for colonization, feeding, and oviposition on plant leaves treated with kaolin and the untreated citrus. There were no significant day or treatment-by-day interactions for nymphs ($P = 0.4$) or adults ($P = 0.19$) in the no-choice tests suggesting that GWSS did not vary over the 4-day period after they had found a suitable site on which to settle (Fig.3ab). Survival of nymphs under no-choice conditions was significantly reduced by kaolin treatments ($P < 0.001$) after being exposed for 4 days, and averaged 8.3 ± 4.2% on kaolin versus 75.0 ± 15.2% on untreated trees. There was no Adult GWSS survival on kaolin-treated lemons and 18.8 ± 4.4% on the untreated lemon 1 day after infestation (Fig.3ab). This relationship did not change over the 4-day period. Adult survival was extremely poor in caged tests because many individuals did not settle on lemon trees under caged conditions and clung to the side of cages until death.

Likewise, the choice tests produced very similar results as the no-choice tests. Neither GWSS nymphs nor adults settled on kaolin-treated lemon, which resulted in no colonization over a 4-day period (Fig.3ab). Treatment preferences of nymphs or adults did not change significantly over time. Survival of nymphs significantly declined over time, from 90.0 ± 4.5% at 1 day to 53.3 ± 6.4% at 4 days after infestation. We hypothesize that this decline in population levels was due to the nymphs falling from PF treated trees or from e movement between treated and untreated foliage and being unable to return to the trees. The number

of surviving adults did not decline in untreated trees in the no-choice test indicating they had favorable conditions to colonize and thrive. Some GWSS nymphs that were able to find the few untreated spots on kaolin-treated foliage probably remained at those sites during the study period. In contrast to the above findings, adult survival on kaolin-treated foliage did not change over time in either the choice or no-choice tests. We observed that kaolin treated plants are undesirable hosts to both GWSS adults and nymphs in choice and no-choice environment.



(a) GWSS nymph counts (b) GWSS adults counts

Fig. 3. Mean number of glassy-winged sharpshooter nymph (n=20) (a) and adults (n=50) (b) per tree 4 days after being released on trees that were treated or not treated with kaolin films in choice versus no-choice experiments.

However, in no-choice experiments, we observed that few nymphs managed to find an untreated site on which to settle when presented with the kaolin-treated plants. This finding implies that good coverage is important when using this material for insect control. When given a choice between kaolin-treated and untreated plants, no GWSS nymphs or adults infested kaolin treated plants.

The choice study may be more representative of what occurs under field conditions because GWSS can utilize more than 75 species of plants (Turner & Pollard, 1959). Therefore, GWSS could easily move to other plants or plant species if they encountered kaolin treated- plants. Results from other studies indicate that kaolin particle films protect plants from insect feeding, oviposition, and by repelling GWSS nymphs and adults by coating the plant surfaces with a mineral barrier (Puterka et al., 2003). This finding is supported by previous reports using kaolin particle film treatments (Glenn et al., 1999; Puterka et al., 2000; Sisterson et al., 2003). There are numerous examples where kaolin particle films repel insect infestations, thus prevent feeding and oviposition although there are at least six other possible mechanisms of action that depend on specific insect-plant relationships (Glenn and Puterka 2005, Puterka and Glenn 2008).

2.2 Mortality effect of kaolin and imidacloprid on GWSS on grape

The mortality effects of kaolin and imidacloprid on GWSS were assessed on grape artificially infested by *X. fastidiosa.* The GWSS was caged for 48 h on grape vines that was previously infested by *X. fastidiosa* (Tubajika et al., 2007). After acquisition, GWSS, in groups

of 10, were caged for 24 or 48 h in small sleeve cages (45 x 55 cm) containing plants either treated or not treated with kaolin and imidacloprid. After 24hr or 48 hr post-treatment, the number of affected GWSS was recorded. The criterion for mortality was insects without any movement (ataxic)[1]. The GWSS mortality was then expressed as percentage.
The GWSS mortality (%) was significantly ($P < 0.05$) impacted by particle film treatment (Tubajika et al., 2007). After 24 hr feeding time, GWSS mortality ranged from 0% (Negative and positive control plants) to 58% (kaolin-treated plants). For the 48 hr feeding time, the mortality ranged from 0% (negative control plants) to 100% (kaolin treated-plants) (Table 1).

	Percent (±SE) Plant mortality[y]	
Treatment\Feeding time	24-hour	48-hour
Kaolin-treated plants	58 ± 14aB	100 ± 25aA
Imidacloprid-treated plants	34 ± 1bB	71 ± 21bA
Positive Control (Infested plants)[x]	0 ± 0cA	9 ± 1cA
Negative Control (Non infested plants)[x]	0 ± 0cA	0 ± 0cA

[x]Untreated plants control plants consisted of water-treated plants exposed or not exposed, respectively to infective GWSS.
[y]Calculated from the means for 5 replications. Within columns (small letters) and across rows (capital letters), means followed by the same letter did not differ significantly ($P < 0.05$) according to Fisher's least significant difference test.

Table 1. Effect of kaolin and imidacloprid treatments and length of glassy-winged sharpshooter (GWSS) feeding time on GWSS mortality in greenhouse experiments.

Overall, the GWSS mortality was greater on imidacloprid-, kaolin-, and the untreated plants when the GWSS fed for 48 h than when they were allowed to feed for 24 h.
However, mortality of the GWSS on untreated control plants and non-infested plants with *X. fastidiosa* was not impacted by the feeding time (negative controls) in these experiments (Table 1). The occurrence of insecticide resistance in GWSS depends on the insecticide used and duration of exposure (Tubajika et al., 2007). Therefore, effective insecticide management strategies and their implementation are necessary for the prevention of rapid resistance development. The mechanism of action of particle films do not rely on toxicity to insects thus resistance to particle films appears unlikely (Puterka & Glenn 2008).

2.3 Pierce's Disease (PD) incidence

Similarly, the impact of kaolin and Imidacloprid on incidence of PD was also assessed on the same grape vines. Each plant was visually assessed at 30-day intervals for PD symptom development which includes stunting, delayed growth, marginal leaf necrosis, and abscission between the petiole and leaf blade. On each assessment date, the total number of plants exhibiting PD symptoms was recorded. Ten leaf samples from each plant were bulked and assayed for the presence of *X. fastidiosa* using assays enzyme-linked immunosorbent assay (ELISA) and immunocapture polymerase chain reaction (IC-PCR)

[1] An inability to coordinate voluntary muscular movements that is symptomatic of some nervous disorders.

(Minsavage et al., 1997)). In determining the incidence of PD, the number of plants with symptoms of PD was expressed as percentage of total plant assessed.
The treatment of plants with kaolin and imidacloprid, exposure time of plants to the infectious GWSS, and the interaction of treatment by exposure time had an effect on PD incidence (Tubajika et al., 2007). PD incidence was 4%, 7%, and 32% in kaolin-, imidacloprid-treated plants, and in untreated controls, respectively (Table 2).

Percent (±SE) Pierce's Disease incidence[yz]		
Treatment\Feeding time	24-hour	48-hour
Kaolin-treated plants	8 ± 2bA	0 ± 0bB
Imidacloprid-treated plants	9 ± 2bA	4 ± 1bB
Positive Control (Infested plants)[x]	48 ± 12aA	19 ± 3aB
Negative Control (Non infested plants)[x]	0 ± 0bA	0 ± 0bA

[x]Untreated plants control plants consisted of water-treated plants exposed or not exposed, respectively to infective GWSS.
[y]Calculated from the means for 5 replications. Within columns (small letters) and across rows (capital letters), means followed by the same letter did not differ significantly ($P < 0.05$) according to Fisher's least significant difference test.
[z]Leaf samples from each plant were assayed for the presence *X. fastidiosa* using ELISA and IC-PCR.

Table 2. Effect of kaolin and imidacloprid treatment and length of glassy winged sharpshooter feeding time on Pierce's Disease incidence.

The incidence of PD did not differ among plants treated with imidacloprid and kaolin. *Xf* detection by ELISA assays was 8%, 11%, and 34% in kaolin-, imidacloprid-treated plants, and the untreated controls, respectively (data not shown). However, incidence of PD was greater when the GWSS were allowed to feed for 24 h versus 48 h. There was no difference in PD incidence on plants treated with imidacloprid and kaolin regardless of the length of the GWSS feeding time. In the untreated control plants, disease incidence was higher in plants exposed to infectious GWSS for 24 h than for 48 h (Table 2). The anti-feedant property of imidacloprid may be important in reducing the acquisition of PD bacterium, decreasing the transmission efficiency of infected GWSS, and subsequently, reducing the spread of PD bacterium (Tubajika et al., 2007). This finding is consistent with results obtained by Krewer et al., (2002), who showed that imidacloprid treatment slowed the development of PD in the field but was not effective in preventing the infection of PD in areas with prevalent sources of inoculum and high vector abundance.

3. Kaolin treatment as a barrier to GWSS movement into vineyard

Previous studies by Blua et al., (1999) and Perring et al., (2001) have shown that GWSS population utilize citrus as their primary reproductive host, and citrus is the predominant overwintering host, when grapes are dormant during winter. The application of insecticide

to control insects can decrease spread and minimize yield loss in certain pathosystems (Purcell & Finley, 1979). Contact insecticides only offer short-term protection against GWSS infestations because of the continued immigration of sharpshooter adults from citrus which re-infest the grapes (Purcell & Finley, 1979).

The prevention of movement of GWSS from citrus to adjacent vineyards by kaolin particle film treatments as barriers was examined. The vineyard, which was comprised of an assortment of Thompson Seedless, Flames Seedless, and Chenin Blanc grape cultivars, was divided into six blocks 164.6 m wide by 365.7 m long (6.5 ha) and assigned treatments of kaolin or conventional insecticides (Puterka et al., 2003). Kaolin barrier treatments only extended 247.5 m into each block; the remaining 152.4 m was left untreated. The insecticide treatment block s had applications the entire 365.7 m distance of the block. Grapes received three bi-weekly applications of kaolin (11.36 kg kaolin, 378.5 per liter water) and six weekly applications of Dimethoate (Dimethoate 400, Platte Chemical Co., Greeley, CO, USA) applied at 4.67 l per ha, methomyl (Lannate LV, E. I. DuPont de Nemours & Co., Wilmington, DE, USA) applied at 2.33 l per ha, and Naled (Dibrom 8E, AMVAC Chemical Corp., Los Angeles, CA, USA) applied at 0.74 l per ha (Puterka et al., 2003). GWSS adults and nymphs were collected from the yellow sticky traps spaced every 30.5 m along the 365.7 m transects per block. Because of edge effect on movement of GWSS from citrus into grapes, plots were partitioned into four distances (0 to 4.3 m (interface); 4.4 to 123.7 m; 123.8 to 247.5 and 247.6 to 365.7 m) to better estimate the number of GWSS. GWSS adults and nymphs will be assessed by weekly monitoring of GWSS adults and nymphs caught per yellow sticky trap (Trece, Salinas, California placed 1 m high in the grape vines in vineyard bordering citrus (interface) and placed every 30.5 m into 365.7 m transects extending into each treatment block (transect) in Kern County, California. GWSS egg masses were sampled by inspecting 25 leaves per vine every 30.5 m along the sticky trap transects in each block.

3.1 GWSS adult counts from traps

Traps in kaolin-tread plants at the edge of vineyard (0-4.3 m) caught fewer GWSS than traps in the insecticide-treated plants based on one experiment from 9 March to 6 April, except on 16 March (Fig. 4a). After 6 April, there were no differences in Interface trap catches between treatments. Transect traps in kaolin barrier treatment resulted in fewer GWSS on 22 March and 6 April. Data from all other trap dates showed that treatment effects were not significantly different. Counts of GWSS adults were significantly different between sample dates, treatments, and blocks (Fig. 4ab).

Visual counts of in the grape-orange grove interface (0-4.3 meters) revealed higher number of GWSS adult in the insecticide treatment than in the kaolin treatment on week 1 (22 March) (kaolin = 0.06 ± 0.06, insecticides = 0.6 ± 0.6), week 2 (29 March) (kaolin = 0.14 ± 0.02, insecticide = 0.56 ± 0.10), and week 3 (6 April) (kaolin = 0.0 ± 0.0, insecticides = 0.9 ± 0.5). Overall, the GWSS adult counts were much lower in traps beyond the particle film barrier in grape (Fig. 4b). The treatment comparison of three applications of kaolin to six insecticide applications over a 2-month period showed that the kaolin barrier was equally effective or better than numerous insecticide applications in reducing GWSS movement into grapes (Puterka et al., 2003). This may appear to be contrary given the fact that the insecticide used was systemic; however, it appears that the kaolin was a sufficient barrier to prevent GWSS movement into the grape vineyards.

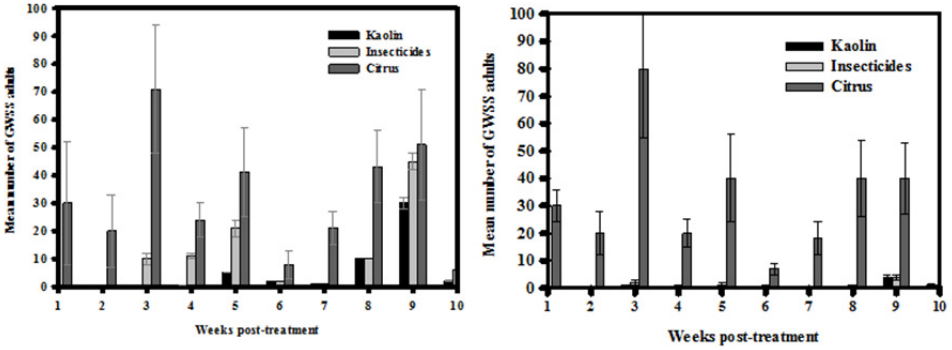


Fig. 4. Effects of kaolin and conventional insecticides on the mean number (±SE) of glassy-winged sharpshooter adults caught per yellow sticky trap (Trece, Salinas, California placed 1 m high in the grape vines in vineyard bordering citrus (interface) and placed every 30.5 m into 365.7 m transects extending into each treatment block (transect) in Kern County, California.

3.2 GWSS egg counts on grape

GWSS egg counts differed significantly (P = 0.0001) between the insecticide and barrier treatments in leaf samples taken by increasing distances from the grape-orange grove interface into the grape vineyard (P = 0.02). The insecticide treatment had significantly (P < 0.0001) larger numbers of GWSS eggs at the edge (0 meters) than at the distances away from the edge of grape leaves in vineyard bordering the citrus (Table 3). This suggest that oviposition on foliage can be affected by proximity to locales of insecticide application.

	GWSS egg counts (±SE)[y]	
Distance from edge of vineyard (m)[x]	Kaolin	Insecticides
0.0 - 4.3 (block 1)[y]	0.00 ± 0.02aA	14.1 ± 5.1aB
4.4 - 123.7 (block 2)	0.06 ± 0.02aA	5.3 ± 2bB
123.8 - 247.5 (block 3)	0.06 ± 0.02aA	1.6 ± 0.3cB
248.6 - 365.7 (block 4)	0.90 ± 0.03bA	1.9 ± 0.2cB

[x]Vineyard was partitioned to monitor the movement of glassy-winged sharpshooter into the grapes. Distance in meters from citrus.
[y]Values are means of 20 observations. Means followed by the same letter did not differ significantly (*P* < 0.05) according to Fisher's least significant difference test.
[z]Interface.

Table 3. Effects of kaolin barrier and conventional insecticides on the numbers ((±SE) of glassy-winged sharpshooter eggs sampled in Bakersfield vineyard bordering citrus from 0 – 365.7 meters from the edge. Yellow sticky traps were monitored from March 9 to May 9 on 25 grape leaves per vine.

In contrast, the oviposition averaged 0.0 to 0.9 GWSS eggs per block in the kaolin barrier treatment and did not differ (P = 0.50) from one another. Overall, the kaolin barrier resulted in undetectable levels of oviposition, whereas insecticides did not prevent oviposition (Puterka et al., 2003). The egg mass sampling has been shown as the best measure of how kaolin barrier treatments and insecticide treatments affected GWSS activity and host suitability (Puterka et al., 2003). This study suggests that unlike other vectors of Pierce's Disease, the GWSS disperse well into vineyard and is able to vector *Xf*, agent causal of PD in grape. This is consistent with the pattern of PD spread that we observed in Kern County, Bakersfield, CA (Tubajika et al., 2004).

4. Impact of imidacloprid on GWSS in citrus

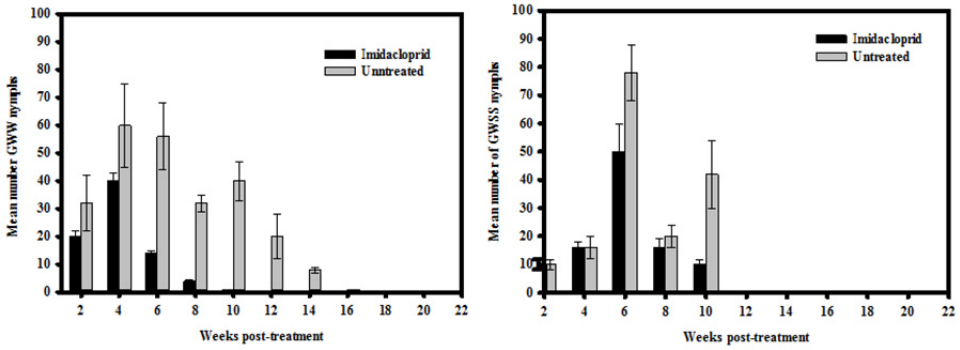
The epidemic of PD in the vineyards of Temecula in Riverside County, California brought into focus the urgent need to control GWSS populations in CA (Castle et al., 2005) around vineyards. Prior to the first applications of imidacloprid which was made in the spring of 2000 for control of GWSS infestations in Temecula, CA; there had been very limited experience with imidacloprid in citrus or against GWSS in any crop (Catle et al., 2005)). Experiments with imidacloprid treatment were carried out at the University of California's Agricultural Operations at Riverside, CA during 2001 and 2002. The experiment was conducted in a block of 10 rows (6.4 m centers) which were split equally between 30 year-old orange trees (var Frost Valencia grafted on Troyer citrange) and lemons (var Lupe grafted on Cook) situated in the center of a 12-ha orchard. The GWSS nymphs and adults were collected in each bag and counted to determine the effect of imidacloprid on GWSS infestations between treated and untreated citrus trees. A sample consisted of four to six rapid thrusts at five locations around each tree. The contents of the collecting jar were then emptied into pre-labeled ziplock bags before moving on to the next tree

4.1 GWSS nymph counts in citrus

Based on the counts in the above experiments, sampling date proved to be a source of variation a two year study. In 2001, ifferences among the three sampling dates from April to June September. GWSS adults and nymphs were significantly ($P < 0.0001$) greater as observed among four dates in 2002 ($P < 0.0001$). Differences in GWSS counts between imidacloprid-treated and untreated trees were observed in 2001 (Fig. 5a) when populations were much larger than in 2002 (Fig. 5b). For the first four weeks following treatment, nymphal counts were high in both treated and untreated trees (Fig 4a). At week 6 post-treatment, a sharp decline in nymphal counts occurred in the imidacloprid-treated oranges coinciding with mean titers of imidacloprid surpassing 5 µg per liter (data not shown). Mean nymphal counts fell to 4.4 (±1.4) by week 8 compared with 30.49 (±4.4) in the untreated control.

The decline on number of nymphs at the beginning of week 9 may be due natural mortality, emigration and emergence to the adult stage, as observed in untreated control. However, mean number of nymphs remained between 30 and 40 through week 10 in the untreated trees while mean counts dropped below 2 at week 10 in the imidacloprid-treated orange trees (Fig 4a). Season-long differences between treated and untreated nymphal counts were significantly ($P < 0.0001$) high based on a repeated measures MANOVA. The decline in nymphal counts in 2001 corresponded nicely with the rise of imidacloprid titers in oranges, but this was not apparent in lemons or during the evaluation the following year when

nymphal counts were so low (Catle et al., 2005). However the variability in the application through the irrigation system and/or the rate of uptake could also accounted for the significant variation in number of nymphs or adults among trees (Catle et al., 2005).



(a) GWSS nymph counts in 2001 (b) GWSS nymph counts in 2002

Fig. 5. Mean (±SE) number of (a) GWSS nymphs in 2001 and (b) in 2002 on oranges trees treated with imidacloprid compared with untreated trees. Sample size each week was n=12 trees using a bucker sampler thrusted a t five different locations per tree.

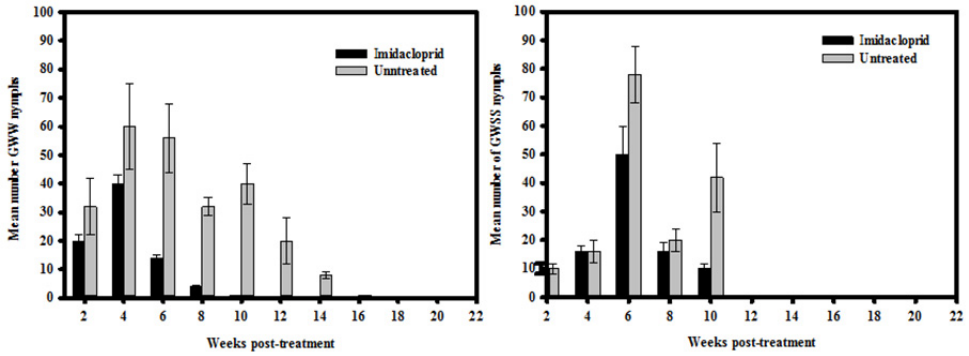
4.2 GWSS adult counts in citrus

GWSS Adults were not observed through the first eight weeks post treatment (Fig 4b). With maturation of the first nymphs and emergence to adults, numbers of adults rapidly increased in both treated and untreated oranges trees between weeks 9 and 12 (15 June–6 July). Contributing to the influx of young adults into the imidacloprid-treated oranges was the absence of any buffer zones between the treated and untreated trees (two rows of treated trees only). By week 14, however, a divergence in the mean number of adults caught in each treatment had begun, reaching its greatest difference in week 18 (17 August). Difference in adult densities was greater throughout week 25 (5 October) after which treated and untreated GWSS adult counts began to converge (Fig 4b). The mean number of GWSS adults between 15 June and 30 November 2001 was significant ($P < 0.0001$) between treated and untreated orange trees.

In contrast to the mean number of nymphs observed in 2001 in orange trees, the difference in number of GWSS nymphs in lemon trees in 2002 was inconsistent treated and untreated lemon trees (Fig 5a). Numbers of GWSS nymphs in untreated lemons were especially erratic, thus making it difficult to observe any clear treatment effect ($P = 0.40$). However, a very similar pattern to the orange trees was observed for GWSS adult counts in treated and untreated lemon trees (Fig 5b).

It is clear that protection by imidacloprid was not sporadic or spatially uneven, but rather was confronted by a phenomenon where mass emergence of adults coupled with heightened flight activity simply overwhelmed both treated and untreated trees in the orchard (Catle et al., 2005). After a few weeks, GWSS adult numbers began to decline and population remained consistently and significantly lower than the untreated orange and lemon trees. Similarly, a rapid increase in nymphal densities occurred in both treated and untreated orange trees in 2001, much as they were observed in Temecula in 2000 (Catle et

al., 2005). The antifeedant effects of imidacloprid on other herbivores belonging to Hemiptera (Sternorrhyncha) are well established (Nauen et al., 1999).



(a) GWSS adult counts in 2001 (b) GWSS adult counts in 2002

Fig. 6. Mean (±SE) number of (a) GWSS adults in 2001 and (b) in 2002 on oranges trees treated with imidacloprid compared with untreated trees. Sample size each week was n=12 trees using a bucker sampler thrusted a t five different locations per tree.

Results from these experiments showed that a recruitment of adults from surrounding orchards during week increased densities in imidacloprid and untreated controls but at levels that were significantly lower for imidacloprid treated–trees. Moreover, Variability in the application through the irrigation system and/or the rate of uptake could have accounted for the significant variation observed among trees. Also, substantial reductions in GWSS nymphs and adults in imidacloprid treated-trees observed during 2001 production year were sustained for 4-5 months post treatment. Overall, GWSS infestations were reduced in imidacloprid-treated trees than in untreated-trees. Data on the number of GWSS adult and nymph counts from grapevines and citrus studies confirmed that citrus is the primary and preferred host for GWSS as observed in Temecula valley and when given other choices such as grape, almond, cherry, stone fruit, the insect feeds on these hosts as Kern County provides a perfect variety of hosts (Tubajika et al., 2007; Blua et al., 1999; Purcell & Saunder, 1999; Raju et al., 1980).

5. Pierce's Disease incidence

Our previous study on the analysis of the spatial patterns of PD incidence in the lower San Joaquin Valley, CA indicated that GWSS may not be infective but their movements within the vineyard after arrival are important in the spread of the infection, which resulted in the symptoms we recorded (Tubajika et al., 2004).

Experimental plots consisting of Thompson Seedless, Flame Seedless and Chenin Blanc cultivars which were approximately 15-years-old were assessed for Pierce's disease (PD) incidence using a visual evaluation of disease symptoms such as stunted shoot growth, leaf scorch, and persistent petioles, a condition that occurs when the leaf blades scald and abscise, leaving only petioles attached to the shoot (Anon, 1992; Purcell, 1974). Plants were assessed at 30-d intervals. Ten leaf samples from each plant were bulked and assayed for the

presence of *X. fastidiosa* using ELISA and IC-PCR and PD incidence was assessed as previously described.

When results were averaged across years, the incidence of PD was significantly ($P < 0.05$) lower (6%) in plots treated with kaolin than in plots treated with conventional insecticides (14%) (Tables 3, 4). There was no significant difference in the incidence of PD among grape cultivars in both years ($P = 0.67$). Also, chemical by cultivar interaction did not affect PD incidence in either year (Tables 3, 4).

Mean (± SE) percent PD incidence[xy]		
Cultivars	Kaolin	Conventional insecticides[z]
Thompson Seedless	8.4 ±1.1 aB	18.7 ± 2.4 aA
Flames Seedless	8.1 ± 1.1 aB	19.2 ± 2.1 aA
Chenin Blanc	7.8 ± 0.5 aB	17.5 ± 2.9 aA
Mean	8.1 ± 0.4	18. 5 ± 2.2

[x]Values are means of eight observations (Four replications x two years). Within rows (small letters) and across rows (capital letters), means followed by the same letter did not differ significantly ($P< 0.05$) according to Fisher's least significant difference test.
[y]Pathogen identity was confirmed by ELISA and IC-PCR assays. *Xylella fastidiosa* strain Temecula (ATCC 700964) collected from grape in Temecula, California was used as a reference control.
[z]dimethoate (Dimethoate 400) applied at 4.67 liter per ha, methomyl (Lannate LV) applied at 2.33 liter per ha and naled (Dibrom 8E) applied at 0.74 liter per ha.

Table 3. Effects of kaolin and conventional insecticides on Pierce's Disease incidence on Thompson Seedless, Flame Seedless, and Chenin Blanc cultivars during the 2001 production year at Bakersfield, CA.

Overall incidence of PD was 43% higher in 2001 than in 2002. All of the infected grape plants were removed from the plots. The inoculum sources for the 2002 season were subsequently reduced (Tubajika et al., 2007). In 2001, PD incidence was 18% on plants treated with insecticides and 4% in kaolin-treated plants (Table3). In 2002; the incidence of PD was 8% on plants treated with insecticides and 4% on kaolin-treated plants (Table 4).

Our previous study on the spatial patterns of incidence of PD in the lower San Joaquin Valley, CA indicates that GWSS may not be infective but their movements within the vineyard after arriving into the vineyard are important in the spread of the infection, and resulted in the symptoms we recorded (Tubajika et al., 2004). Field studies showed that plants treated with kaolin were less likely to become infected with *X. fastidiosa* and had a lower incidence of PD symptoms than untreated control plants (Tubajika et al., 2007). There are limited reports on application of particle film to control plant diseases caused by the vector population in the fields (Glenn et al., 1999; Blua et al., 1999; Puterka et al., 2000; Puterka et al., 2003). Also, data showed that the GWSS had a lower rate of survival following exposure to kaolin-treated plants for 48 h. This finding is similar to previous reports where kaolin completely protected plants from insect feeding (Blua et al., 1999; Puterka et al., 2000; Puterka et al., 2003). They suggested that the kaolin protects hosts against GWSS by camouflaging the plant with a white coating making them visually unperceivable, or by reflecting sunlight, which repels leafhoppers as well as aphids.

Mean (± SEM) percent PD incidence[xy]		
Cultivars	Kaolin	Conventional insecticides
Thompson Seedless	4.3 ±0.5 aB	8.8 ± 1.4 aA
Flames Seedless	4.2 ± 0.5 aB	8.1 ± 1.1 aA
Chenin Blanc	3.9 ± 0.2 aB	7.9 ± 0.9 aA
Mean	4.1 ± 0.3	8.3 ± 1.3

[x]Values are means of eight observations (Four replications x two years). Within rows (small letters) and across rows (capital letters), means followed by the same letter did not differ significantly ($P < 0.05$) according to Fisher's least significant difference test.
[y]Pathogen identity was confirmed by ELISA and IC-PCR assays. *Xylella fastidiosa* strain Temecula (ATCC 700964) collected from grape in Temecula, CA was used as a reference control culture.
[z]dimethoate (Dimethoate 400) applied at 4.67 liter per ha, methomyl (Lannate LV) applied at 2.33 liter per ha and naled (Dibrom 8E) applied at 0.74 liter per ha.

Table 4. Effects of kaolin and conventional insecticides on Pierce's Disease incidence on Thompson Seedless, Flame Seedless, and Chenin Blanc cultivars during the 2002 production year at Bakersfield, CA.

6. Conclusion

The GWSS was recently introduced into California and poses a serious threat to the grape industry because it is a very effective vector of the bacterium that causes Pierce's disease. This introduction of the GWSS has changed the epidemiology of *Xf* diseases affecting important agronomic and horticultural crops as well as landscape ornamental and native trees in CA by infesting these crops with the bacteria. The epidemic of PD in the vineyards brought into focus the urgent need to control GWSS populations, especially around vineyards. A new technology, called particle film and a systemic insecticide, imidacloprid were assessed both in greenhouse and field.

Results showed that kaolin protects plants from insect feeding, oviposition, and infestation by coating the plant surfaces with a protective mineral barrier. In caged field studies, we found that GWSS nymphs and adults were highly repelled by lemon trees treated with kaolin. In field studies that compared three biweekly kaolin treatments to six weekly contact insecticide treatments, kaolin performed as well as insecticides in reducing GWSS adult numbers and oviposition. A good coverage of plant leaves with kaolin is important when using this material for insect control. In greenhouse studies, GWSS adult counts were reduced greatly in kaolin-treated plants versus untreated control trees.

Based on data on GWSS nymph and adults counts in citrus, the persistence of imidacloprid in citrus varied as near-peak levels of imidacloprid were sustained for 6–10weeks before gradually declining as substantial reductions in GWSS nymphs and adults were observed in imidacloprid-treated trees during the 2001 trial and were sustained for 4–5months post-treatment. Imidacloprid effect on GWSS nymphs was not as well pronounced in the 2002 trial, when overall GWSS infestations were much reduced from the previous year. However, consistently lower adult infestations were observed in 2002 for imidacloprid compared with untreated trees.

The application of kaolin and imidaclorprid impacted GWSS mortality and PD incidence. Higher GWSS mortality rates were observed on kaolin-treated plants than on untreated-plants. The reduction in PD incidence observed in field studies suggest that either kaolin treatments as barriers to GWSS infestation or imidacloprid can be effective in reducing GWSS population and PD symptoms, and can be valuable tools for PD management. Both treatments strategies could also be used where insect resistance to imidacloprid would be a concern since insect resistance to particle films would not be a concern. Additionally, these treatments could be combined with other PD management approaches in integrated pest management.

The costs of chemicals and applications by either commercial applicators or growers were not determined in these studies. However, the choice of chemical to apply (alone or in combination) is important and the level of net returns will depend on this as well as other factors such as frequency of applications, disease intensity, and growth stage at which the chemical sprays are initiated. Additional work assessing these and other potential benefits are needed to fully determine the economic value of these treatments for grape production.

7. References

Almeida, R. P. P., & Purcell, A. H. (2003). Transmission of *Xylella fastidiosa* to grapevines by Homalodisca coagulata (Hemiptera cicadellidae). J. Econ. Entomol. 96, 264–271.

Anon. (1992). *Grape pest management, 2nd ed. Univ. of California, Div. Nat. Res., Okland, CA. Publ. No. 3343*.

Blua, M. J., Phillips, P. A., & Redak, R. A. (1999). A new sharpshooter threatens both crops and ornamentals. *Calif. Agric., 53*, 22-25.

Castle, S. J., F.J, B., Jian, B. L., & Toscan, N. C. (2005). Spatial and temporal distribution of imidacloprid and thiamethoxam in citrus and impact on *Homalodisca coagulata* populations. . *Pest Manag. Sci, 61*, 75-84.

Costa, H. S., Blua, M. S., Bethke, A., & Redak, R. A. (2000). Transmission of *Xylella fastidiosa* to oleander by the glassy-winged sharpshooter, *Homalodisca coagulata*. HortScience 35, 1265–1267.

Glenn, D. M., Puterka, F. G., van der Zwet, T., Byers, R. E., & Feldhake, C. (1999). Hydrophobic particle films: a new paradigm for suppression of arthropod pests and plant diseases. J. Econ. Entomol. 92, 759–771.

Glenn, D., and G. J. Puterka. (2005) Particle Films: A new technology for Agriculture. Pp. 1-44. In J. Janick [ed.] HortReviews. Vol.31. John Wiley & Sons, Inc., Hoboken, NJ.

Hemingway, J., & Ranson, H. (2005). Chemical Control of Vectors and Mechanisms of Resistance. In W. C. Marquardt (Ed.), *Biology of Disease Vectors* (second ed., pp. 627-637). San Francisco: Elsevier Academic Press.

Hill, B. L., & Purcell, A. H. (1995). Multiplication and movement of *Xylella fastidiosa* within grape and four other plants. Phytopathology 85, 1368–1372. .

Krewer, G., Dutcher, J. D., & Chang, C. J. (2002). Imidacloprid 2F insecticide slows development of Pierce's disease bunch grapes. J. Entomol. Sci. 37, 101–112. .

Minsavage, G. V., Thompson, C. M., Hopkins, D. L., Leite, B. C., & Stall, R. E. (1994). Development of a polymerase chain reaction protocol for detection of *Xylella fastidiosa* in plant tissue. Phytopathology 84, 456–461. .

Nauen, R., Koob, B., & Elbert, A. (1999). Antifeedant effects of sublethal dosages of imidacloprid on *Bemissia tabaci*. *Entomol. Exp. Appl., 88*, 287-293.

Perring, T. M., Farrar, C. A., & Blua, M. J. (2001). Proximity to citrus influences Pierce's disease in Temecula Valley vineyards. Calif. Agric. 55, 13–18.

Pooler, M. R., Myung, I. S., Bentz, J., Sherald, J., & Hartung, J. S. (1997). Detection of *Xylella fastidiosa* in potential insects by immunomagnetic separation and nested polymerase chain reaction. Lett. Appl. Micro¬biol. 25, 123–126.

Purcell, A. H. (1974). Spatial patterns of Pierce's disease in the Napa Valley. Am. J. Enol. Viticutt. 25, 162–167. .

Purcell, A. H., & Finley, A. H. (1979). Evidence for noncirculative transmis¬sion of Pierce's disease bacterium by sharpshooter vectors. Science 206, 839–841.

Purcell, A. H., Saunders, R. S., Hendson, M., Grebus, M. E., & Henry, M. J. (1999). Causal role of *Xylella fastidiosa* in Oleander leaf scorch disease. Phytopathology 89, 53–58.

Purcell, A. H., & Saunders, S. R. (1999). Glassy-winged sharpshooters expected to increase plant disease. Calif. Agric. 53, 26–27.

Puterka, G. J., Glenn, D. M., Sekutowski, D. G., Unruh, T. R., & Jones, S. K. (2000). Progress toward liquid formulations of particle films for insect and disease control in pear. Environ. Entomol. 29, 329–339.

Puterka, G. J., Reinke, M., Luvisi, D., Ciomperlik, M. A., Bartels, D., Wendel, L., et al., (2003). Particle film, Surround WP, effects on glassy-winged sharpshooter behavior and its utility as a barrier to sharpshooter infestations in grapes. Plant Health Prog.Online, doi: 10.1094/PHP-2003-0321-RS. .

Puterka, G. J., and D. M. Glenn. (2008) Kaolin-Based Particle Films for Arthropod Control. pp. 2075-2080, In (J. L. Capinera, Ed.) Encyclopedia of Entomology, Kluwer Academic Publishers.

Raju, B. C., Nome, S. F., Docampo, D. M., Gohen, A. C., Nyland, G., & Lowe, S. K. (1980). Alternative hosts of Pierce's disease of grapevines that occur adjacent to grape growing areas in California. Am. J. Enol. Viticult. 31, 144–148.

Romoser, W. S., & Stoffolano, G. J. (1998). *The Science of Entomology.* Boston, MA: WCB/McGraw-Hill.

Sisterson, M. S., Liu, Y. B., Kerns, D. L., & Tabashinik, B. A. (2003). Effects of kaolin particle film on oviposition, larval mining, and infestation of cotton by pink bollworm (Lepidoptyera: Gelechiidae). *J. Econ. Entomol., 96*(3), 805-810.

Sorensen, S. J., & Gill, R. J. (1996). A range extension of *Homalodosca coagulata* (Say) (Hemiptera: Clypeorrhyncha: Cicadellidae) to southern California *Pan-Pac Entomol., 72*, 160-161.

Tubajika, K. M., Civerolo, E. L., Ciomperlik, M. A., Hashim, J. M., & Luvisi, D. A. (2004). Analysis of the spatial patterns of Pierce's disease incidence in the lower San Joaquin Valley in California. Phytopathology 94, 1136–1144

Tubajika, K. M., Civerolo, E. L., Puterka, G. J., Hashim, J. M., & Luvisi, D. A. (2007). The effects of kaolin, harpin, and imidacloprid on development of Pierce's disease in grape *Crop Protection, 26*, 92-99.

Turner, W. F., & Pollard, H. N. (1959). Life histories and behavior of five insect vectors of phony peach disease. USDA Techn. Bull.1188.

Unruh, T. R., Knight, A. L., Upton, J., Glenn, D. M., & Puturka, G. J. (2000). Particle-film for suppression of the codling moth in apple and pear orchards. *J. Econ.Entomol., 93*, 737-743.

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Use and Management of Pesticides in Small Fruit Production

Carlos García Salazar[1], Anamaría Gómez Rodas[2] and John C. Wise[2]
[1]*Agriculture & Agribusiness Institute,*
Michigan State University Extension, West Olive, MI
[2]*Department of Entomology, Trevor Nichols Research Center, Fennville, MI*
USA

1. Introduction

Historically, pesticide management has been an inefficient process for pest control in tree fruit production. The amount of active ingredient released into the pest habitat is thousands of times greater than the amount of pesticide required to kill it if the treated population were confined in a jar or a small space. To illustrate this, lets take the oral LD50 for methyl parathion in rats (18 to 50 mg/kg) (1, 2) and the field dose for controlling the Brown Stink Bug (BSB) in pecans (Penncap-M, 227 grams of active ingredient (AI) per acre) (3). If the weight of the population of BSB in one acre of pecans were the equivalent of 1 kg, taking the LD50 of 50 mg/kg for methyl parathion we may need only 100 mg to kill the entire population if treated in a jar or a small confined space. But since they are distributed all over the field it takes thousand times more product than needed to kill every insect present in the treated field.

In today's world, due to the increased cost of the pesticides a more efficient approach is needed, as well as more potent poisons requiring smaller application rates and new environmental legislations. In 1996, the enactment of the Food Quality Protection Act (FQPA) in the United States of America changed for good the way we controlled pests in agriculture in general (4). The enactment of the FQPA legislation demanded the re-registration of all pesticides used in agriculture until 1996. One of the major changes was the introduction of a 10 X safety margin for children for pesticides shown to have a deleterious effect on children and woman during pregnancy.

The most important changes occurring under FQPA were that pesticides had to be evaluated in relation to the aggregated exposure taking into consideration all potential sources of exposures to residues in food, water and residential use. Pesticides with a common mode of action potential exposure had to be assessed as a cumulative exposure. Finally, potential risk must be assessed without considering the benefits.

Organophosphate (OP) or conventional insecticides used extensively in fruit production were some of the most affected by FQPA. This created the need for new chemicals to substitute those products that were removed from the list of approved materials. Products that were not cancelled required mitigation measures that restricted their use. These substitute pesticides are less effective and require multiple applications to obtain similar results than the ones obtained with conventional OP's. This difference in the performance of the new pesticides increased the cost of pest control. New products are more pest-specific, a

small amount of a.i. is required per application (< 1 oz /acre) and intensive site-specific IPM pest scouting is required to effectively use them.
Under this new paradigm, an effective use and management of pesticides require growers and IPM practitioners to know the chemistry of the pesticide being used, the biology of the pest and its behavior, the influence of weather, plant structure, and equipment used to release the pesticide into the pest habitat.

2. Problems associated with the use of pesticides in agriculture

Every year there are a number of claims to farm insurance companies that are the result of errors during the application of pesticides. The main causes for losses of crops due to misapplications are equipment failure (24%), improper tank mixes (33%) and drift (33%). Other causes are application in the wrong field and problems reading the label of the product (5). The most important main factors affecting the efficacy of pest control are, the prevailing weather conditions during the application, the type of nuzzles and conditions of the spray equipment, the characteristics of the crop and the behavior of the pest. Although the applicator can not control some of these factors there are always measures that may help reduce the pesticide environmental impact and increase their effectiveness.

3. Weather conditions during the application

Wind velocity. It is usually the most critical factor of all meteorological conditions affecting the efficacy of the pesticide application. The greater the wind speed, the farther off-target a droplet of a given size will be carried. The larger the droplet, the less it is affected by the wind and the faster it will fall. High winds however, can cause even larger droplets to move off-target. Therefore, spraying operations should be stopped if wind speeds are excessively high (> 10 miles/hour or 16 Km/hour). In 2001, the US Environmental Protection Agency (EPA) issued a recommendation that indicated that for pesticide applications in orchards and vineyards and other fruit crops the maximum wind speed during the application should be between 3 and 10 miles/hour (4.5-16 Km/hour) (EPA 730-N-01-006). To illustrate the effect of wind velocity on pesticide droplets we may take the data from Rose and Lambi (1985) (6). They showed that droplets 100 microns in diameter travel 4.6 m in a wind speed of 1.6 Km/hour. However, in a wind speed of 8 Km/hour they travel 23.1 m. A droplet of 400 microns under the same conditions travels 1 and 5 m, respectively.
Relative Humidity. As droplets fall through the air, they evaporate into the atmosphere. This evaporation reduces the size and mass of the particle enabling it to remain airborne longer and, under the right conditions, to drift farther from the application site. The rate at which water evaporates from the spray particles depends primarily on air temperature and relative humidity. At 70 % relative humidity and 78°F, a 100-micron droplet will fall 5 feet and hit the ground before evaporating to half its original diameter. However, at 30% relative humidity and 78°F, a 100-micron droplet quickly evaporates and becomes one-eighth of its original volume while falling only 2.5 feet. While evaporative loss of spray materials occurs under almost all atmospheric conditions, these losses are less pronounced under the environmental conditions that occur during the cooler parts of the day - early morning and late afternoon. The relative humidity is usually highest during these cooler periods (7).
Atmospheric stability. It is an important factor influencing drift. Under normal (stable) meteorological conditions, the air temperature decreases by 5.4°F per 1,000 feet of height (1°

C per 100 meters). Cool air tends to sink, displacing lower warm air and causing vertical mixing. As a warm air layer rises, suspended droplets rise with it and dissipate into the upper layers by normal air turbulence and vertical mixing. Vertical mixing is the result of the ADIABATIC effect. Adiabatic effect refers to the behavior of a parcel of air when it moves up and down through a gradient of pressure in the atmosphere—this being greatest at the surface and diminishes with height. This process occurs as follows: **Rising Air Parcel.** This movement causes *adiabatic cooling*. As the air parcel rises it enters a low pressure region and this causes the parcel to expand. This sudden expansion causes a decrease in the parcel's temperature and it starts cooling down. This effect is similar to opening a canister of propane gas, while it escapes the gas forms a frosty cap at the tip of the exit. **Falling Air Parcel.** This creates the *adiabatic warming*. As the parcel of air starts falling down, it enters a region of high pressure. This change in atmospheric pressure compresses the parcel of air and causes an increase in its temperature. The parcel of air continues descending until its pressure and temperature equals the surrounding air (Figure 1).

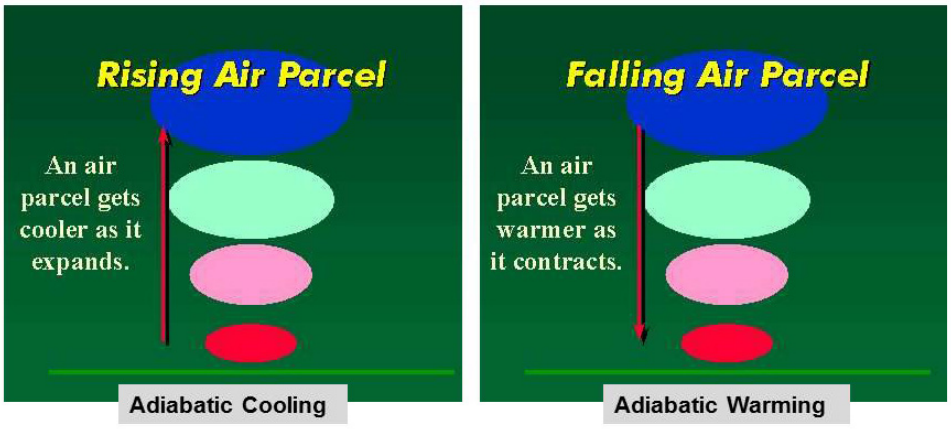

Fig. 1. Adiabatic effect.

When the rising parcel of air gets cooler than the surrounding air, it stops rising. This produces a situation in which the temperature at ground level and the temperature several hundred meters above the ground are the same. There is no vertical movement of the air and the atmosphere is in a condition of stability. Conversely, if the rising parcel gets warmer than the surrounding air, it continues rising creating the condition of atmospheric instability where parcels of air move up and down as they warm up and cool down.

Movement of pesticide droplets. When water-encapsulated pesticide droplets exit the nozzle their final deposition depends on the droplet size, the air and water temperature, and the environmental relative humidity. Big droplets (400-500 microns) will reach the foliage before losing substantial amount of volume. But all other droplets that do not reach the foliage are affected by the adiabatic effect. When those droplets are falling to the ground they are compressed by the atmosphere, their temperature rises beyond the surrounding air and they become airborne until their temperature equals the surrounding air. As those droplets cool down they fall back and the process is repeated again (Figure 2).

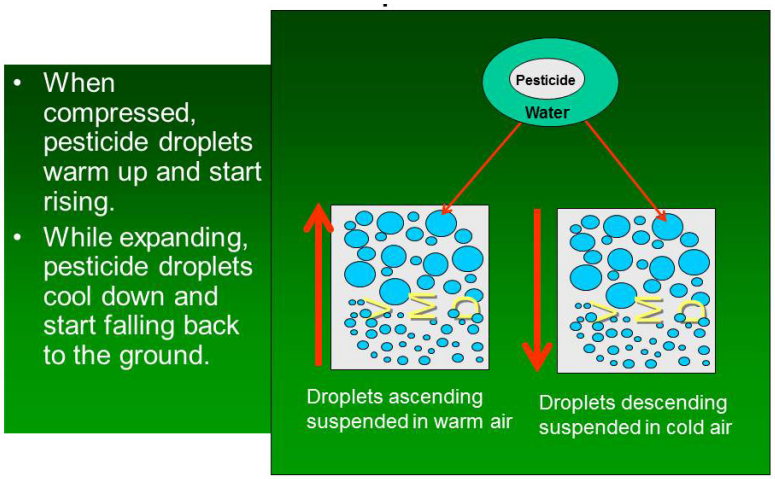

Fig. 2. Adiabatic effect on droplets containing the pesticides as they exit the nuzzle.

4. Spray application under unstable conditions

As warm air rises, suspended droplets rise with it and dissipate into the upper layers by normal air turbulence and vertical mixing. Under these conditions, the opportunity for crop injury at any specific off target site is very small because the pesticide is dispersed and diluted into the atmosphere. **Figure 3**, shows the application with a cannon sprayer in blueberries under unstable conditions. The picture shows that most of the drift remains confined to the first 5-6 rows facing the sprayer and the cloud of droplets that constituted drift moves up with little horizontal movement out of the target area

Fig. 3. Spray application under unstable conditions in a blueberry field.

5. Spray application under stable conditions

Under stable air conditions since the temperature at ground level is the same over the next few hundred meters above the ground, vertical air movement is hindered and there is little or no atmospheric mixing. Consequently, fine droplets will hang in the air and slowly diffuse superficially on relatively level terrain.

Fig. 4. Spray application early in the morning with no vertical mixing.

In Figure 4 the effect of atmospheric stability is presented. Fine spray droplets remain aloft traveling horizontally away from the target area. In this picture, pesticide drift goes over the road falling on passing by vehicles. Applications under these circumstances are the most problematic because they cause dangerous drifts that end up creating environmental pollution.

A very common type of atmospheric stability is the one created by an inversion of the gradient of temperature occurring in the atmosphere. Under conditions of instability the air temperature decreases with an increase in altitude, 5.4°F per 1,000 feet of height (1° C per 100 meters, approximately). Very often the air temperature near the ground is cooler than the air a few meters above ground; the surrounding air gets warmer with increasing altitude. This creates a layer of hot air that acts like a cap that does not allow the vertical movement of the air holding down cooler air underneath. This phenomenon is what we call thermal inversion and is very frequent very early in the morning and during the evening hours when the ground cools the air layer immediately above it.

Spray application under thermal inversion conditions. Pesticide applications conducted under conditions of thermal inversion are prone to produce large quantities of dangerous drifts. Pesticide particles suspended in the cool layer cannot move anywhere except laterally, possibly for several miles. **Do not spray** during thermal inversions, when air close to the ground is colder than the air above it. You should not spray early in the morning in still air if sensitive areas are nearby (especially down gradient if the terrain is sloped). Applicators concerned about these adverse spraying conditions should wait until late

afternoon or early evening to spray, when there is less chance of the atmosphere being inverted and conditions are more favorable.

6. Spray equipment used for pesticide applications in small fruit crops

The most important factor in providing an efficient pesticide cover is type of equipment. The efficacy of your pest control depends on the right selection of your spraying equipment. Different types of spraying equipment are used in small fruit crops for pest control. Air-blast spraying equipment is the most common equipment employed for pesticide application (Figure 5).

Fig. 5. Air assisted sprayers: right, a cannon sprayer; left, conventional air-blast sprayer.

However, when the crop canopy makes it difficult to apply pesticides using ground equipment fixed-wing aircrafts are mainly used (Figure 6).

Fig. 6. Aerial application of insecticide in blueberries near harvest with fixed-wing aircrafts (Fennville, Michigan 2006).

Different air-assisted equipment produces different types of spray which in turn have different distribution on the target crop. Table 1 shows the properties of different air-assisted equipment on the deposition of pesticides on the target canopy.

Application Technology	Droplet size	Canopy Penetration	Field Distribution
Air-blast Sprayer	Fine – Very Coarse	High	Uniform
Cannon Sprayer	Very Fine – Medium	Medium	Horizontally-Variable
Aerial Application	Fine – Medium	Medium	Vertically Variable
Proptec Sprayer	Fine – Medium	High	Uniform

Table 1. Pesticide application equipment and characteristics of spray deposition.

Show (1996) gave a very good definition of every type of spray depending on the droplet size (5). Accordingly, a very fine spray (VF) is made of droplets with a diameter < 182 microns (μm); A fine spray (F) is made of droplets with a diameter ranging from 183 to 280 μm; Medium spray (M) is made of droplets ranging from 281 to 429 μm; A coarse spray (C) is made of droplets ranging from 430 to 531 μm in diameter, etc.
The type of spray produced by the equipment determines the volume of water needed to spray the recommended dose of pesticide. The following table indicates how much water is needed to cover one acre of blueberries using different types of sprays.

Spray type	Volume type	Droplet diameter (μm)	Application rate gal/acre
Coarse	High	500	108
Medium	Medium	200 - 500	32 -108
Fine	Low	100 - 200	6 - 32

Table 2. Type of spray and its effect on the amount of water needed to spray one acre of blueberries.

7. Plant structure and canopy: where do your chemicals end up?

After selecting the right environmental conditions and the equipment for the spray application the next factors determining the success of your applications will be, type of canopy and plant structure.
Plant structure is important in determining the final destination of your spray. It also influences the success of pest control. Canopy is the place where the pest is found scouting for food, mating, looking for oviposition sites or escaping from natural enemies and adverse environmental factors. This also includes escaping from the applications of pesticides. Therefore, in determining the amount of water and the equipment required to control a pest problem, type of target crop and plant structure are crucial.
In blueberries, for example, the structure of the bush is important in the efficacy of the application of insecticides and fungicides using air blast sprayers. A well pruned bush will allow for better penetration of the pesticide. Thus, you could achieve better pest control and

savings in product. On the other hand, thick canopies prevent the placement of the product in the habitat of the pest resulting in poor and more expensive pest control. Van Ee et al.(2000) demonstrated how different types of pruning affected pesticide deposition in "highbush" blueberries (*Vaccinium coymbosnm* L) at different times during the plant growth season. Their goal was to determine how sprayer type, pruning severity, and canopy development interacted to affect spray deposition patterns. Deposition was measured as the percentage of the surface area of card targets that was covered following applications of black dye. Light measurements indicated that the canopy of blueberry bushes, regardless of pruning treatment, closed by the middle of June, and light levels within the canopy changed little from then until fruit harvest in August. They used a standard airblast sprayer that pushed spray up and white kromcoat cards clipped to each target. Targets were clipped to branches in a random orientation in the same two bushes used for the light measurements. Figures 7.1, 7.2, and 7.3 showed the conditions of pruning of treated bushed before treatments were applied.

Fig. 7.1. Lightly pruned bushes.

Fig. 7.2. Moderately pruned bushes.

Fig. 7.3. Heavily pruned bushes.

Van Ee, found that spray penetration and deposition in all types of canopies was greater in the bush's side facing the sprayer. However, spray penetration and coverage was greatly reduced at the far side of the bush when the field was lightly pruned. Conversely, medium or heavy pruning allowed more penetration and deposition than in light pruned bushes. Moderate or heavy pruning allowed similar deposition, except at the top of the bush. In the heavily pruned bushes pesticide deposition was greater than at any other canopy structure (7.3).

In 2006, we conducted a study to determine the spray deposition patterns in mature blueberries using different spray equipment. A mature Jersey field was sprayed with Surround WP (kaolin clay) utilizing a cannon sprayer, an airblast sprayer and a 20-gallon aerial application (Wise et al. unpublished results).

We found that a spray application of 50 gal/acre in light pruned bushes resulted in high deposition at the bottom and middle portion of the canopy and less deposition on the top of the bush. The same volume applied with a cannon sprayer resulted in higher spray deposition on top of the bush but less deposition on the middle section and much less at the bottom of the bush.

The application of a spray volume of 20 gallons to the same field with fixed-wing aircraft resulted in an even deposition of the spray from top to bottom of the bush.

These results were very important to establish what type of equipment we need to use to apply pesticides in blueberry fields in order to optimize the efficacy of the pesticide.

8. Pest habitat and behavior

Finally, the purpose of the spray application is to place the pesticide in contact with the pest. This objective can be achieved in two ways; 1) depositing the pesticide in the habitat of the pest were the pest may enter in contact with the pesticide or 2) placing the pesticide in contact with the pest by taking advantage of its behavior. In this case the pest "finds" the product while moving around the canopy of the crop.

For plant diseases and insects that do not move a lot and remain confined to a small area (a leaf, branch, bush or section of the canopy) we need to bring the pesticide to the pest by targeting their habitat. Spray cover has to be very good with enough number of droplets per square centimeter of foliage to maximize the probability of the pest getting in contact with the spray. In the case of blueberry aphids and fruitworms, pest control measures require placing the insecticide in the habitat of the pest. Blueberry aphids live at the bottom, inside

of the bush. Thus, spray applications should reach the bottom of the bush. If the product applied is systemic, droplet size does not really matter, the product will be translocated into de plant and the insect will acquire the lethal dose while sucking up sap. With the Cranberry fruitworm (*Acrobasis vaccinii* Riley) and the Cherry fruitworm (*Grapholita packardi* Zeller), if pest control is directed against eggs and larvae it should be directed into the calyx of the fruit. The product needs to be placed also into the calyx where larvae will hatch. In this case a large volume of spray needs to be deposited on the top of the blueberry bush where the fruit is produced and the fruitworms mate and deposit their eggs. In both cases, a large number of small droplets of pesticide need to be applied into the habitat of the insect.
With an insect or pest that moves a lot or explores intensively the canopy of the crop, like the Blueberry Maggot (*Rhagoletis mendex*) or the Cranberry fruitworm first instar larvae. A few large droplets will be enough to have a good control of these pests. Adult blueberry maggot explores intensively the blueberry canopy in search of mates, food and sites to oviposit. Also, first instar Cranberry fruitworm larvae after hatching crawl out of the berry calix to search for a place to enter the berry at the site where the peduncle and the fruit meet. In both insects the wandering period is critical to put the pesticide in contact with the pest. Table 3 presents a summary of the habitat and behavior of several insects that attack blueberries in Michigan.

Pest	Targeted Life Stage	Pest Behavioral Activity Level	Location on the Plant	Exposure Period
Fruitworms	Egg/larva	Low	Fruit Cluster	Short
Leafrollers	Larva	Medium	Upper Canopy	Long
Blueberry Maggot	Adult	High	Upper Canopy	Short
Japanese Beetle	Adult	High	Upper Canopy	Long
Blueberry Aphid	Adult/Nymphs	Low	Lower Canopy	Long
Bud mites	Adult/Nymphs	Low	Buds	Short

Table 3. Habitat and behavior of several insect pest of "Highbush" blueberries.

9. Pesticide management summary

We can summarize the recommendations for optimizing the efficacy of the pesticides as follow:
The pesticide efficacy and efficiency will depend on:
Weather conditions that affect the movement of pesticides in the environment.
- Avoid spraying under low relative humidity,
- Avoid spraying when wind speed is greater than 10 mph,

- Avoid spraying under stable or thermal inversion conditions

Equipment. This affects pesticide deposition in the target site (amount of product and percentage of surface area covered).

- Use the appropriate spraying equipment,
- Calibrate your sprayer,
- Use the appropriate spray volume.

The conditions of the sprayer will limit the biological effect of the spray. Important factors to consider are droplet size, volume and distribution of the application

Crop conditions. This affects the penetration of chemicals and pesticide deposition. Proper pruning facilitates:

- spray penetration and deposition,
- and placement of the pesticide into the pest habitat,
- removes refuge sites where the pest can find shelter.

In the case of crop conditions, the canopy density limits the spray's penetration and deposition. It will be more difficult to implement a successful pesticide management program in a slightly pruned field than in a field that is properly pruned. A thick canopy provides many places where the pest can be protected from natural enemies and from our pesticides! Under these circumstances, only a limited amount of our spray will reach the pest habitat limiting the effectiveness of our pest control program.

Pest behavior. Each pest species has its particular behavior. Therefore, the efficacy of your pest control will depend on:

- your understanding of the pest biology and behavior,
- the behavior of a pest that limits the critical window to control it

Knowing the pest is important because it is the objective of our pest control program. If we do not understand the pest biology and behavior, chances are we will miss the critical times when the pest is more susceptible to our pesticides.

Finally, the success of your **Pesticide management program will depend on** your ability in putting the pesticide in contact with the pest either by:

- Placing the pesticide in the pest habitat and/or,
- Taking advantage of the pest behavior

10. References

[1] Hayes, W.J. and E.R. Laws (ed.). 1990. Handbook of Pesticide Toxicology, Vol. 3, Classes of Pesticides. Academic Press, Inc., NY.

[2] Meister, R.T. (ed.). 1992. Farm Chemicals Handbook '92. Meister Publishing Company, Willoughby, OH.

[3] Crop Data Management Systems, Inc. 2007. PennCap-M. http://www.cdms.net/LDat/ld403009.pdf

[4] U.S. Food and Drug Administration. 2009. The Food Quality Protection Act of 1996. http://www.fda.gov/RegulatoryInformation/Legislation/FederalFoodDrugandCosmeticActFDCAct/SignificantAmendmentstotheFDCAct/ucm148008.htm.

[5] Show, B.W. 1996. Minimizing Spray Drift. Texas Agricultural Extension Service, The Texas A&M University System. College Station, Texas. agsafety.tamu.edu/Programs/Ag-Chemical/drift.ppt

[6] Ross, Merrill A. and Carole A. Lembi. 1985. Applied Weed Science. Burgess Publishing Company, Minneapolis, MN.
[7] H. Erdal Ozkan. 1998. Effect of Major Variables on Drift Distances of Spray Droplets. The Ohio State University Extension (AEX-525-98). http://ohioline.osu.edu/aex-fact/0525.html.
[8] Van Ee, G., Richard Ledebuhr, Eric Hanson, Jim Hancock, and Donald Ramsdell (2000) Canopy Development and Spray Deposition in Highbush Blueberry. HortTechnology 10(2) 354-357.

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The Conundrum of Chemical Boll Weevil Control in Subtropical Regions

Allan T. Showler
USDA-ARS, Kika de la Garza Subtropical Agricultural Research Center, Weslaco, Texas U.S.A.

1. Introduction

Originally a Mesoamerican insect, the boll weevil, *Anthonomus grandis grandis* Boheman (Coleoptera: Curculionidae), has spread from the tropics, where it evolved on cotton, *Gossypium hirsutum* L., and other malvaceous plant species (Burke et al., 1986; Brubaker & Wendel, 1994; Showler, 2009b), north to temperate cotton producing areas of the United States and south to northern provinces of Argentina (Cuadrado, 2002; Showler, 2009b). The pest was first detected in United States cotton in 1892 (Parencia, 1978) and infested the Cotton Belt such that by 1917, every cotton-producing county in Georgia, for example, was infested (Hunter, 1917). Adults oviposit inside cotton buds or "squares" (usually one egg per square), and the hatched larva causes the square to abscise before it can flower (Showler and Cantú, 2005). If an egg is deposited within a young boll (older bolls become too hard to penetrate), or if mouthparts penetrate the rind of squares to the inner reproductive portion, fiber-producing locks can be injured or completely destroyed, but not necessarily all four locks (Showler, 2006a; Showler & Cantú, 2008). Boll weevil losses have been valued at $83.34 billion and insecticide-based control costs at $18.67 billion between 1893 and 1999, and infestations became so injurious that cotton-free winter periods were instituted by law in some areas (Haney, 2001). Later, insecticide-based eradication programs were launched in the United States and in Argentina (Dickerson & Haney, 2001; Haney et al., 2001; Johnson & Martin, 2001; Texas Department of Agriculture, 2002; Carmona et al., 2003).

Natural enemies indigenous to the United States are not considered to be important as mortality factors against boll weevils (Jones & Sterling, 1979; Showler & Greenberg, 2003), although the imported fire ant, *Solenopsis invicta* Buren, native to South America (Buren et al., 1974; Lofgren, 1986), can account for up to 58% of boll weevil mortality in relatively wet regions where the predator thrives (Sturm & Sterling, 1990). In one study imported fire ant predation on immature boll weevils averaged 84% compared with 0.14% and 6.9% mortality caused by parasitism and desiccation, respectively (Fillman & Sterling, 1983). But in drier cotton growing areas, lack of sufficient predation to help govern populations in some new habitats outside Mesoamerica (Showler, 2007) permitted rapid dispersal (Burke et al., 1986; Showler, 2009a). While certain cultural practices, such as early planting (Showler et al. 2005) can help avoid large populations that typically accumulate in the summer (Showler, 2003, 2005), chemical intervention against building infestations has been the chief control tactic.

In subtropical south Texas, before the boll weevil eradication program was re-instated in the fall of 2005 (after a halted attempt in 1995), crop protection against boll weevils was approached using three tactics: mandatory cotton stalk destruction before 1 September, prohibition on planting until 1 February and elimination of volunteer cotton during the cotton-free winter period (Texas Department of Agriculture, 2002). Insecticides were the only in-season control approach (Showler, 2007). Some growers sprayed 2–3 "pre-emptive" treatments starting at the "pinhead" square size (1–2-mm diameter) (Heilman et al. 1979) followed by insecticide applications (often azinphosmethyl, cyfluthrin, bifenthrin, or oxamyl) whenever 10% of randomly selected medium (3–5.4-mm diameter) or large (5.5–8-mm diameter) squares (Showler, 2005; Showler et al., 2005) had oviposition punctures (Showler et al., 2005). While Heilman et al. (1979) suggested that pre-emptive spraying delays insecticide applications later into the season, other research found no beneficial effect (Showler, 2004a) and the economic value of the practice is debatable. Pre-emptive sprays might kill some adult boll weevils that have entered the field after overwintering elsewhere, but the sparse numbers of weevils at that time and the presence of less-preferred and nutritionally inferior small squares contribute relatively little to field-level population buildups, and injury to such small squares has negligible impact on lint yield (Showler, 2004b; Showler et al., 2005). Late-season spraying for immediate crop protection (not eradication) purposes is also of questionable utility because, although feeding and oviposition punctures can be abundant on bolls, older bolls (≥14 days old) are less vulnerable to attack (because they harden) than younger (≤10 days old) bolls and bolls do not abscise in response to boll weevil oviposition (Showler, 2006a). When injury to a boll does occur, usually because of prior adult feeding during the square stage or larval infestation of the boll, damage is often limited to individual lint-bearing locks, of which there are four (Showler 2006a). Insecticides applied in the context of crop protection after cut-out (Guinn, 1986; Cothren, 1999), when bolls predominate, generally fail to measurably suppress boll weevil infestations (Showler & Robinson, 2005; Showler, 2008a).

When cotton is forming medium and large squares, which are most vulnerable to and useful for boll weevil reproduction (Showler, 2004b), the 10% spray intervention threshold is compromised by variability in total numbers of squares over time (Showler, 2007). Declining abundance of squares coupled with surges in boll weevil populations contribute toward the likelihood of triggering interventions based on randomly sampled squares; hence, spraying later protects fewer and fewer squares (Showler, 2007). A better estimate of infestation would involve comparing numbers of oviposition-punctured squares to total squares within, for example, three-meter (or some other length) sections of rows (Showler, 2007). In a study in south Texas, the standard approach, including three pre-emptive sprays, involved nine applications that failed to increase yield and economic return (Showler & Robinson, 2005). Once 10% of the squares harbor a boll weevil egg, protecting it from contact insecticides (Showler & Scott, 2004), it is too late to expect good control.

In temperate areas of the United States, the boll weevil eradication program has had remarkable successes since its beginning in North Carolina and Virginia in 1978 to Georgia to California (Dickerson et al., 2001) and the pest has been eradicated from northern and central regions of Texas as well (USDA-APHIS, 2007; Texas Boll Weevil Eradication Foundation, 2011). The boll weevil is "functionally" eradicated in other areas of Texas (USDA-APHIS, 2007), whereby <0.001 weevils/trap/week were found during the most recently completed growing season, indicating that boll weevils are not reproducing or causing economic damage in an area [*e.g.*, >1.5 million ha of cotton in 2010 (Texas Boll

Weevil Eradication Foundation, 2011)]. Eradication involves three techniques over a 4–5-year period: pheromone trap detection, reduction of boll weevil food supply, and malathion treatments (USDA-APHIS, 2007). The process starts with a series (often seven) of fall malathion applications that were once [and probably erroneously (Showler, 2010)] termed "diapause sprays" (McKibben et al., 2001). The aim is to reduce late season populations that will be further, and dramatically, reduced during cold temperate winters when food is unavailable and temperatures can be lethal (Showler, 2009b,c, 2010). Pheromone traps are then deployed around all cotton fields during the following spring planting and spraying of each field begins 5–6 weeks later based on trap captures; the process is repeated annually until the boll weevil is no longer found (USDA-APHIS, 2007). Still, there are ≈262,000 ha of cotton in east and subtropical south Texas where eradication has not been achieved (Texas Boll Weevil Eradication Foundation, 2011). While the chance of boll weevil dispersal from infested areas on wind and in hurricane systems exists (Texas Boll Weevil Eradication Foundation, 2011), particularly from nearby Mexico where eradication efforts are not underway, there are other overriding factors contributing to the pest's persistence in the subtropics (Showler, 2007, 2009b). Misconceptions relating to boll weevil ecology and biology (Showler, 2009c), while immaterial in cold temperate areas, appear to underlie the challenges to eradication efforts under subtropical conditions (Showler, 2009b, 2010).

2. Misunderstandings

There have been misconceptions pertaining to fundamental aspects of boll weevil survival outside its native Mesoamerican region that involve dietary habits, overwintering, and diapause, all of which interrelate (Showler, 2007, 2009b,c, 2010), presenting obstacles to temperate eradication approaches when used in the subtropics. Ultimately, the problem resides in numbers of boll weevils (including offspring from overwintering weevils) that can survive cotton-free winters to feed and reproduce in large cotton plantings of the following season. In south Texas, large end-of-season populations can be observed by trapping at the edges of cotton fields that are disrupted by defoliant application, consequent host plant desiccation, harvest, and stalk shredding (Showler, 2003). Those populations move into surrounding habitats where, under temperate winter conditions, the boll weevils that survived the first-year series of late-season eradication program sprays must survive frequently severe and extended cold conditions for which the tropical insect had not evolved, as well as starvation due to lack of viable winter plant hosts (Showler, 2009b,c). Boll weevils have long been assumed to feed solely on pollen of certain malvaceous plants (Burke & Earle, 1965; Cate & Skinner, 1978), and later, pollens of other plants were recognized (Jones et al., 1992, 1993; Hardee et al., 1999), but recent research has revealed that adult boll weevils can consume cotton leaves and bracts, citrus and cactus fruit, and likely nectar (Showler & Abrigo, 2007; Showler, 2009b). In the subtropics, adult boll weevils can survive and reproduce during the winter on small patches of volunteer cotton that, despite surveillance, are overlooked, and adults can be trapped in substantial numbers around grapefruit, *Citrus paradisi* Macfad., and orange, *C. sinensis* (L.) Osbeck., orchards (Showler, 2006b). The edible endocarps of grapefruits and oranges of those citrus species can sustain up to 25% of adult boll weevils in nonreproductive condition for longer than five months (completing the cotton-free period); the maximum longevity (246 days) was only seven days less than boll weevils fed large cotton squares (Showler & Abrigo, 2007). The fruit of prickly pear cacti, *Opuntia* spp. [114 species in Mexico alone (Vigueras & Portillo, 2001)], which is

widespread and abundant in south Texas, can support 10% of adult boll weevils over the winter period, and there are likely other as yet unreported food sources (Showler, 2009b). Hence, in subtropical areas of North and South America where cotton is grown in proximity with citrus, persistent boll weevil populations have been reported even after cotton growing was eliminated or where eradication programs have begun (Cuadrado, 2002; Carmona et al., 2003; Mas et al., 2007; Texas Boll Weevil Eradication Foundation, 2011). Despite the availability of *Opuntia* spp. and other host plants in Mexico and south Texas (Gaines, 1935; Lukefahr, 1956; Lukefahr & Martin, 1962; Stoner, 1968; Cross et al., 1975; Vigueras & Portillo, 2001), cotton in the Lower Rio Grande Valley remained free of boll weevils for 30 years of commercial production beginning ≈1860 (Garza & Long, 2001) even though cotton around Monclova, Coahuila, Mexico, ≈45 minutes latitude north and 220 km west of the Lower Rio Grande Valley, was so heavily infested that the crop was abandoned in 1862 (Howard, 1897). Boll weevil food sources under orchard conditions are concentrated and support substantial active populations through winter (Showler, 2006b) because endocarps are accessible through cracks, holes, or lesions while the fruit is attached to the plant or fallen (Showler, 2007; Showler & Abrigo, 2007). Establishment of boll weevils in Lower Rio Grande Valley cotton during the early 1890s (Parencia, 1978; Haney, 2001) may have been connected to a simultaneous citrus industry boom (Waibel, 1953). The author has witnessed, in mid January, large flying populations of boll weevils in and around nonsanitized (fallen fruit on the orchard floor not removed) orange and grapefruit orchards in south Texas that were so abundant that they were a nuisance. Boll weevils are also known to reproduce in volunteer cotton during Lower Rio Grande Valley winters (Summy et al., 1988), which also contradicts widely accepted, but apparently erroneous, dogma regarding the existence of winter diapause (Showler, 2009c, 2010).

For more than 50 years, boll weevils have been assumed to enter a state of winter diapause (Brazzel & Newsom, 1959), but diapause-induction studies involved weak experimental methods and dubious interpretations of results, and recent research in the subtropics indicates that boll weevils, being of tropical origin, did not evolve a diapause mechanism for surviving temperate winters (Showler, 2007, 2009c, 2010). Sterling and Adkisson's (1966) finding that boll weevils in the Texas High Plains "diapause" earlier and in greater percentages than in Central Texas (at a lower latitude) implies that boll weevil dormancy is not seasonal (a criterion for diapause), but it is instead responsive to dormancy-triggering conditions whenever they occur (Showler, 2010). Brazzel and Newsom (1959), however, claimed that, in the instance of boll weevils, diapause could be a "facultative" response to harsh, unfamiliar, conditions such as cold temperate winters. It is more likely, however, that the response is merely a metabolic and locomotory slowing caused by declining temperature (Fye et al., 1969; Jones & Sterling, 1979; Watson et al., 1986), giving the appearance of being facultative. As winter temperatures cool, a threshold for quiescence (Koštál, 2006; Guerra et al., 1984) or some other nondiapause expression of dormancy is reached first, followed later, if temperatures become sufficiently cold, by mortality (Showler, 2010). Whatever words are employed to describe the insect's response to temperate winters, eradication strategy involving "diapause spraying" has been effective where temperate winter attrition is substantial even if "diapause" might not be the technically correct term (Showler, 2010).

The inescapable point is that under subtropical conditions, particularly in the presence of relatively large plantings of citrus throughout the agricultural landscape, boll weevil mortality is not as great as in cold-winter temperate areas because winters are generally

warm and can support populations with food until the spring (Showler, 2009b). In February, when cotton can be planted, boll weevil numbers near south Texas citrus orchards were found to be substantial (Showler, 2006b). Loss of major winter attrition as an eradication tool will likely require adjustments to the customary approach. Chance movement of boll weevils on wind or farm vehicles into active eradication program areas might cause setbacks to eradication, but the ecological reasons for the boll weevil's persistence in subtropical areas presents broader and more difficult challenges.

3. Chemical tactics: no easy answers

3.1 Insensitive trigger

The spray regimen for cotton crop protection against boll weevils and the reasons it was sometimes not sufficient across all growing areas have been discussed, but aspects of eradication involving insecticide application are also weakened in the subtropical context. Monitoring in-season boll weevil populations, for example, is important for determining whether to intervene and to assess efficacy. It is surprising that boll weevil surveillance fails to account for in-season changes in adult boll weevil response to grandlure largely predicated by cotton plant phenology and associated volatiles. One change occurs as cotton begins to square; then, even while boll weevils are accumulating in cotton fields, few are collected in the traps (Parajulee et al., 2001), presumably a result of competing plant volatiles from large fields of cotton versus a point pheromone source. Further, the trap's physical design presents a series of obstacles that boll weevils must negotiate before finding their way into a plastic cap on top where the weevils are counted (Showler, 2007). At low ambient populations in south Texas, differences in numbers of boll weevils captured in the conventional trap versus a sticky board trap were not detected, both traps using the same pheromone lure, but at higher populations sticky board traps collected ≥9-fold more weevils than the conventional trap, and 30% of the conventional traps collected no boll weevils when corresponding sticky boards accumulated from 82 to 511 weevils at the same locations and time; on one occasion, the conventional trap had two boll weevils compared with 2,228 on a sticky board (Showler, 2003). This is not to suggest that sticky board traps should replace the conventional trap unless their deployment can be made less labor intensive, but a more sensitive trap design would refine spray timing for greater effect as a result of more accurate population detection.

3.2 Spray timing

Because the boll weevil's life cycle includes ≈18 days in immature life stages protected within squares (Showler & Cantú, 2005), commonly-used insecticides with relatively short residual effects (≤4 days) can miss that cohort (Showler & Scott, 2004). To ensure lethal exposure to a larger proportion of the population, such insecticides would have to be sprayed *at least* once every four days. Yield increases in experimental plots were reported where some were sprayed "proactively" every 7–8 days starting when ≈2% of randomly selected squares were large (Showler & Robinson, 2005). It is unlikely, however, that the proactive spray regime would be as effective in larger commercial fields on an area-wide scale; in the study, applications were meticulous and tractor-mounted drop nozzles provided complete coverage even when the plants were high. For large boll weevil populations likes those encountered in the Lower Rio Grande Valley (Showler 2003), insecticides would have to be applied every three or four days from the time medium-sized

squares (3–5.4-mm- diameter) first develop (before 2%) until cut-out when square production declines rapidly (Guinn, 1986; Cothren, 1999).
Under subtropical field conditions, feeding on pinhead- and match-head-sized squares is negligible, and large squares are preferred to medium-sized squares regardless of planting date (Showler, 2005). Boll weevil feeding punctures on large squares were 7.8- and 25-fold more abundant compared with match-head squares and bolls, respectively (Showler, 2004b). In terms of nutritional value, medium and large squares promote greater egg production and longevity of adult boll weevils than any other stage of cotton fruiting body (Showler, 2008b), and in terms of providing enough food and space for the immature stages of the boll weevil to develop, pinhead and match-head squares are generally too small (Showler, 2004b). Hence, spraying insecticides well before medium and large square sizes are available is of little value to crop protection and for impeding boll weevil reproduction, which agrees with the recommendation by Norman and Sparks (1998) for beginning boll weevil control in the Lower Rio Grande Valley when one-third-grown squares appear. Once large squares blossom and form post-bloom, young, and hardened older bolls, the nutritional value for longevity and egg production declines to nil when the rind can no longer be penetrated (Showler, 2004b). This explains why adult boll weevil populations plateau following cut-out through harvest (Showler et al., 2005). While spraying during the late season, particularly the series of late season eradication program sprays that occur in the first year (USDA-APHIS, 2007), can likely reduce boll weevil numbers, warm winters with plentiful food can ensure the survival of many until after spring cotton planting.
Scott et al. (1998) reported that, in the Lower Rio Grande Valley, early- and medium-maturing cotton varieties produce the best yields. In a similar vein, square production in early-planted cotton is lower than in later plantings and avoids the high numbers of weevils occurring in later-planted cotton (Showler et al., 2005). Although late-planted cotton produces more squares than early-planted cotton, this advantage is off-set by losses from heavy boll weevil infestations (Showler et al., 2005). The best time for planting was found to be intermediate between early and late for an optimal balance between increasing square production while avoiding the greatest accumulations of boll weevils, thereby reducing insecticide applications as well (Showler et al., 2005).
Harvest timing can also influence insecticide use. From a crop protection perspective, although harvesting late (at 75% boll splitting) rather than earlier (at 40% boll split) can require an extra insecticide application where using the proactive approach, particularly when boll weevil populations were relatively large, but harvesting late captures greater quantities of lint when more bolls have matured, resulting in better economic return, even if the late season insecticide treatment is superfluous (Showler & Robinson, 2008). Mixing the defoliant with an insecticide was found to be relatively ineffective and unreliable (Showler, 2008a).

3.3 Resistance

Boll weevil tolerance to organophosphorus, carbamate, and pyrethroid insecticides was reported by Kanga et al. (1995), but analyses of field populations have not detected resistance to malathion. It is conceivable, however, that under continual insecticide pressure from malathion only, resistance might develop (Bottrell et al., 1973), and because the boll weevil eradication program relies exclusively upon malathion, exposed boll weevil populations should be assessed intermittently for signs of resistance. For the time being, malathion remains toxic to boll weevils, even at reduced rates (Showler et al., 2002).

4. Possibilities

There are a number of ways in which chemical boll weevil control might be improved. First, a more sensitive trap would permit increasingly timely responses to the early in-season presence of adult boll weevils (but not while squares are still match-head size). At that point, spraying should provide continuous protection of vulnerable and nutritious medium- and large-sized squares. Even if sprays occur weekly, achieving acceptable control on an area-wide scale is improbable, which suggests that using a more sensitive trap design could result in more appropriately-timed, and likely increased, spray applications for the subtropics (unless spraying is conducted at ≤4-day intervals between late match-head to cut-out stages) where overwintering populations are relatively large (Showler, 2007). Both crop protection strategies and the eradication approach should evolve to incorporate emerging information on boll weevil ecology to find tactics that can help mitigate population buildups, such as avoiding late planting, use of earlier-maturing varieties, and development of longer-residual insecticides to reduce numbers of applications and to enhance protection of squares. Because subtropical boll weevil populations are active during winter and can sustain themselves on citrus, removal of such plentiful food through post-harvest orchard sanitation would augment the ban on cotton. Another overlooked tactic is plant resistance. While cotton has been bred for a variety of traits, no cultivars have been developed to resist boll weevil attack. Efforts in this direction might include altering square rind thickness or consistency to make the inner portion, where the immature weevil stages develop, less accessible, or changing the availability of certain nutrients that can affect egg production (Showler, 2009a). Eradicating a tropical pest like the boll weevil in temperate areas was achievable, but the subtropics are more akin to the insect's native habitat in terms of temperature and host plants. For this reason, adjustments to the temperate eradication strategy might have to involve tailoring insecticides, application timing, and the circumstances under which they are applied (*e.g.*, as influenced by planting dates and phenological stages of the crop) for extending prophylactic crop protection and decimating boll weevil populations as selectively as possible to avoid the possibility of for secondary pest outbreaks.

However, even were all of the issues surrounding subtropical boll weevil eradication to be resolved, the feasibility of remaining boll weevil-free in areas along international borders is compromised by boll weevil populations breeding on the other side of the border where attention to eradication, for a complex of reasons, may not be in synchrony. Hence, the success of eradication in subtropical border areas depends to a great extent on the coordinated efforts of both countries. In the instance of a somewhat analogous pest, the desert locust, *Schistocerca gregaria* (Forskål), which can move long distances as massive swarms in Africa and the Middle East, breeding in one country can put crops in neighboring countries at risk, resulting in perpetually reactive and increasingly insecticide-based, rather than preventive maintenance strategies (Steedman, 1988; Showler & Potter 1991; Showler, 1995).

5. References

Bottrell, D.G.; Wade, L.J. & Bruce, D.L. (1973). Boll weevils fail to develop resistance to malathion after several years of heavy exposure in Texas High Plains. *Journal of Economic Entomology* 66: 791-792, ISSN 0022-0493.

Brazzel, J.R. & Newsom, L.D. (1959). Diapause in *Anthonomus grandis* Boh. *Journal of Economic Entomology* 52: 603-611, ISSN 0022-0493.

Brubaker, C.L. & Wendel, J.F. (1994). Reevaluating the origin of domesticated cotton (*Gossypium hirsutum*; Malvaceae) using nuclear restriction fragment length polymorphisms (RFLPs). *American Journal of Botany* 81: 1309-1326, ISSN 00029122.

Buren, W.F.; Allen, G.E.; Whitcomb, W.H.; Lennartz, F.E. & Williams, R.N. (1974). Zoogeography of the imported fire ant. *Journal of the New York Entomological Society* 82: 113-124, ISSN 0028-7199.

Burke, H. R.; Clark, W.E.; Cate, J.R. & Fryxell, P.A. (1986). Origin and dispersal of the boll weevil. *Bulletin of the Entomological Society of America* 32: 228-238, ISSN 1046-2821.

Burks, M.L. & Earle, N.W. (1965). Amino acid composition of upland cotton squares and Arizona wild cotton bolls. *Journal of Agricultural and Food Chemistry* 13: 40–43, ISSN 0021-8561.

Carmona, D.; Huarte, M.; Arias, G.; López, A.; Vincini, A.M.; Castillo, H.A.; Manetti, P.; Capezio, S.; Chávez, E.; Torres, M.; Eyherabide, J.; Mantecón, J.; Cichón, L. & Fernández, D. (2003). Integrated pest management in Argentina. In: *Integrated Pest Management in the Global Arena*, K. M. Maredia, D. Dakouo & D. Mota-Sanchez, (eds.), pp. 303-326, CABI Publishing, ISBN 0-85199-652-3, Wallingford, England.

Cate, J.R. & Skinner, J.L. (1978). The fate and identification of pollen in the alimentary canal of the boll weevil. *Southwestern Entomologist* 3: 263-265, ISSN 0147-1724.

Cothren, J.T. (1999). Physiology of the cotton plant. In: *Cotton: Origin, History, Technology, and Production*, C.W. Smith (ed.), pp. 207-268. John Wiley and Sons, ISBN 9780471180456, New York, New York, USA.

Cross, W.H.; Lukefahr, M.J.; Fryxell, P.A. & Burke, H.R. (1975). Host plants of the boll weevil. *Environmental Entomology* 4: 19-26, ISSN 0046-225X.

Cuadrado, G.A. (2002) *Anthonomus grandis* Boheman (Coleoptera: Curculionidae) in central and southwest area of Misiones, Argentina: pollen as feeding source and their relationship with the physiological state in adult insects. *Neotropical Entomology* 31: 121-132, ISSN 1519-556X.

Dickerson, W.A. & Haney, P.B. (2001). A review and discussion of regulatory issues. In: *Boll Weevil Eradication in the United States Through 1999*, W.A. Dickerson, A L. Brashear, J T. Brumley, F.L. Carter, W.J. Grefenstette, & F.A. Harris (eds), pp. 137-155. The Cotton Foundation, ISBN 0939809060, Memphis, Tennessee, USA.

Dickerson, W.A.; Brashear, A.L.; Brumley, J.T.; Carter, F.L.; Grefenstette, W.J. & Harris F.A. (2001). *Boll Weevil Eradication in the United States Through 1999.* The Cotton Foundation, ISBN 0939809060, Memphis, Tennessee, USA.

Fillman, D.A. & Sterling, W.L. (1983) Killing power of the red imported fire and [Hym.: Formicidae]: a key predator of the boll weevil [Col.: Curculionidae]. *Entomophaga* 28: 339-344, ISSN 0013-8959.

Fye, R.E.; McMillian, W.W.; Hopkins, A.R. & Walker, R.L. (1959). Longevity of overwintered and first generation boll weevils at Florence, S.C. *Journal of Economic Entomology* 52: 453-454, ISSN 0022-0493.

Gaines, R.C. (1934). The development of the boll weevil on plants other than cotton. *Journal of Economic Entomology* 27: 745-748, ISSN 0022-0493.

Garza, A.A. & Long, C. (2001). *The Handbook of Texas Online*. ww.tsha.utexas.ede/handbook/online/ articles/CC/hcc4.html.

Guerra, A.A.; Garcia, R.F.; Bodegas, P.R. & De Coss, M.E. (1984). The quiescent physiological status of boll weevils (Coleoptera: Curculionidae) during the noncotton season in the tropical zone of Soconusco in Chiapas, Mexico. *Journal of Economic Entomology* 77: 595-598, ISSN 0022-0493.

Guinn, G. (1986). Hormonal relations during reproduction. In: *Cotton Physiology*, J.R. Mauney & J.M. Stewart (eds.), pp. 113-136. The Cotton Foundation, ISBN 0939809-01-X, Memphis, Tennessee, USA.

Haney, P.B. (2001). The cotton boll weevil in the United States: impact on cotton production and the people of the Cotton Belt. In: *Boll Weevil Eradication in the United States Through 1999*, W.A. Dickerson, A.L. Brashear, J.T. Brumley, F.L. Carter, W.J. Grefenstette, and F.A. Harris (eds). The Cotton Foundation, ISBN 0939809060, Memphis, Tennessee, USA.

Haney, P.B.; Herzog, G. & Roberts, P.M. (2001). Boll weevil eradication in Georgia. In: *Boll Weevil Eradication in the United States Through 1999*, W.A. Dickerson, A.L. Brashear, J.T. Brumley, F.L. Carter, W.J. Grefenstette, and F.A. Harris (eds), pp. 259-289. The Cotton Foundation, ISBN 0939809060, Memphis, Tennessee, USA.

Hardee, D.D.; Jones, G.D. & Adams, L.C. (1999). Emergence, movement, and host plants of boll weevils (Coleoptera: Curculionidae) in the Delta of Mississippi. *Journal of Economic Entomology* 92: 130-139, ISSN 0022-0493.

Heilman, M.D.; Namken, L.N.; Norman, J.W. & Lukefahr, M.J. (1979). Evaluation of an integrated production system for cotton. *Journal of Economic Entomology* 72: 896-900, ISSN 0022-0493.

Howard, L.O. (1897). Insects affecting the cotton plant. *USDA Farmers' Bulletin* No. 47: 1-31.

Hunter, W.D. (1917). The boll weevil problem with special reference to means of reducing damage. *USDA Farmers' Bulletin* No. 848: 1-40.

Johnson, D.R. & Martin, G. (2001). Boll weevil eradication in Arkansas. In: *Boll Weevil Eradication in the United States Through 1999*, W.A. Dickerson, A.L. Brashear, J.T. Brumley, F.L. Carter, W.J. Grefenstette, and F.A. Harris (eds), pp. 225-233. The Cotton Foundation, ISBN 0939809060, Memphis, Tennessee, USA.

Jones, D. & Sterling, W.L. (1979). Manipulation of red imported fire ants in a trap crop for boll weevil suppression. *Environmental Entomology* 8: 1073-1077, ISSN 0046-225X.

Jones, R.W.; Cate, J.R.; Hernandez, S.M. & Navarro, R.T. (1992). Hosts and seasonal activity of the boll weevil (Coleoptera: Curculionidae) in tropical and subtropical habitats of northeastern Mexico. *Journal of Economic Entomology* 85: 74-82, ISSN 0022-0493.

Jones, R.W.; Cate, J.R.; Hernandez, E.M. & Sosa, E.S. (1993). Pollen feeding and survival of the boll weevil (Coleoptera: Curculionidae) on selected plant species in northeastern Mexico. *Environmental Entomology* 22: 99-108, ISSN 0046-225X.

Kanga, L.H.B.; Plapp, F.W., Jr.; Wall, M.L.; Karner, M.A.; Huffman, R.L.; Fuchs, T.W.; Elzen, G.W. & Martinez-Carrillo, J.L. (1995). Monitoring tolerance to insecticides in boll weevil populations (Coleoptera: Curculionidae) from Texas, Arkansas, Oklahoma, Mississippi, and Mexico. *Journal of Economic Entomology* 88: 198-204, ISSN 0022-0493.

Koštál, V. (2006). Eco-physiological phases of insect diapause. *Journal of Insect Physiology* 52: 113-127.

Lofgren, C. S. (1986). History of imported fire ants in the United States. In: *Fire Ants and Leaf-cutting Ants: Biology and Management*, C.S. Lofgren & R.K. Vander Meer (eds.), pp. 36-47. Westview Press, ISBN 9780813370712, Boulder, Colorado, USA.

Lukefahr, M.J. (1956). A new host of the boll weevil. *Journal of Economic Entomology* 49: 877-878, ISSN 0022-0493.

Lukefahr, M.J. & Martin, D.F. (1962). A native host plant of the boll weevil and other cotton insects. *Journal of Economic Entomology* 55: 150-151, ISSN 0022-0493.

Mas, G.E.; Grilli, M.P. & Ravelo, A. (2007). Esudio de la dinámica publacional del picudo del algodonero (Anthonomus grandis Boheman) mediante un sistema de información. http://www.geogra. uah.es/inicio/web_11_confibsig/PONENCIAS/2-035-Mas-Ravelo-Grilli.pdf

McKibben, G.H.; Villavaso, E.J.; McGovern, W.L. & Grefenstette, B. (2001). United States Department of Agriculture – research support, methods development and program implementation. In: *Boll Weevil Eradication in the United States Through 1999*, W.A. Dickerson, A L. Brashear, J T. Brumley, F.L. Carter, W.J. Grefenstette, & F.A. Harris (eds), pp. 101-135. The Cotton Foundation, ISBN 0939809060, Memphis, Tennessee, USA.

Norman, J.W., Jr. & Sparks, A.N., Jr. (1998). Managing cotton insects in the Lower Rio Grande Valley. *Texas Agricultural Extension Service Bull. 1210*, Texas A&M University, College Station, TX.

Parajulee, M.N.; Slosser, J.E.; Carroll, S.C. & Rummel, D.R. (2001). A model for predicting boll weevil (Coleoptera: Curculionidae) overwintering survivorship. *Environmental Entomology* 30: 550-555, ISSN 0046-225X.

Parencia, C.R., Jr. (1978). One hundred twenty years of research on cotton insect in the United States. *USDA Agricultural Handbook* No. 515: 1-17: 62-68.

Scott, A.W. Jr.; Lukefahr, M.J.; Cook, C.G.; Spurgeon, D.W.; Raulston, J.R. & Kilgore, T.P. (1998). The strategy of delayed planting with rapid fruiting cotton for the Lower Rio Grande Valley of Texas. In: *Proceedings of the Beltwide Cotton Conferences*, National Cotton Council, pp. 578-579. Memphis, Tennessee, USA, ISSN 0032-0889.

Showler, A.T. & Potter, C.S. (1991). Synopsis of the 1986-1989 desert locust (Orthoptera: Acrididae) plague and the concept of strategic control. *American Entomologist* 37: 106-110, ISSN 1046-2821.

Showler, A.T. (1995). Locust (Orthoptera: Acrididae) outbreak in Africa and Asia, 1992-1994: an overview. *American Entomologist* 41: 179-185, ISSN 1046-2821.

Showler, A.T.; Sappington, T.W.; Foster, R.N.; Reuter, K.C.; Roland, T.J. & El-Lissy, O.A. (2003). Aerially applied standard rate malathion against reduced rates of malathion + cottonseed oil for boll weevil control. *Southwestern Entomologist* 27:45-58, ISSN 0147-1724.

Showler, A.T. (2003). Effects of routine late-season field operations on numbers of boll weevils (Coleoptera: Curculionidae) captured in large-capacity pheromone traps. *Journal of Economic Entomology* 96: 680-689, ISSN 0022-0493.

Showler, A.T. & Greenberg, S.M. (2003). Effects of weeds on selected arthropod herbivore and natural enemy populations, and on cotton growth and yield. *Environmental Entomology* 32: 39-50, ISSN 0046-225X.

Showler, A.T. (2004a) Assessment of pre-emptive insecticide applications at pinhead square size for boll weevil control. In: *Proceedings of the Beltwide Cotton Conferences*,

pp. 1689-1692. National Cotton Council, Memphis, Tennessee, USA, ISSN 0032-0889.

Showler, A.T. (2004b). Influence of cotton fruit stages as food sources on boll weevil (Coleoptera: Curculionidae) fecundity and oviposition. *Journal of Economic Entomology* 97: 1330-1334, ISSN 0022-0493.

Showler, A.T. & Scott, A.W., Jr. (2004). Effects of insecticide residues on adult boll weevils and immatures developing inside fallen cotton fruit. *Subtropical Plant Science* 56: 33-38, ISSN 1009-7791.

Showler, A.T. (2005). Relationships of different cotton square sizes to boll weevil (Coleoptera: Curculionidae) feeding and oviposition in field conditions *Journal of Economic Entomology* 98: 1572-1579, ISSN 0022-0493.

Showler, A.T. & Cantú, R.V. (2005). Intervals between boll weevil (Coleoptera: Curculionidae) ovipostion and square abscission, and development to adulthood in Lower Rio Grande Valley, Texas, field conditions. *Southwestern Entomologist* 30: 161-164, ISSN 0147-1724.

Showler, A.T. & Robinson, J.R.C. (2005). Proactive spraying against boll weevils (Coleoptera: Curculionidae) reduces insecticide applications and increases cotton yield and economic return. *Journal of Economic Entomology* 98: 1977-1983, ISSN 0022-0493.

Showler, A.T.; Greenberg, S.M.; Scott, A.W. Jr. & Robinson, J.R.C. (2005). Effects of planting dates on boll weevils (Coleoptera: Curculionidae) and cotton fruit in the subtropics. *Journal of Economic Entomology* 98: 796-804, ISSN 0022-0493.

Showler, A.T. (2006a). Boll weevil (Coleoptera: Curculionidae) damage to cotton bolls under standard and proactive spraying. *Journal of Economic Entomology* 99: 1251-1257, ISSN 0022-0493.

Showler, A.T. (2006b). Short-range dispersal and overwintering habitats of boll weevils (Coleoptera: Curculionidae) during and after harvest in the subtropics. *Journal of Economic Entomology* 99: 1152-1160, ISSN 0022-0493.

Showler, A.T. (2007). Subtropical boll weevil ecology. *American Entomologist* 53: 240-249, ISSN 1046-2821.

Showler, A.T. & Abrigo, V. (2007). Common subtropical and tropical nonpollen food sources of the boll weevil (Coleoptera: Curculionidae). *Environmental Entomology* 36: 99-104, ISSN 0046-225X.

Showler, A.T. (2008a). Efficiency of tank-mixing insecticide with defoliant against adult boll weevil (Coleoptera: Curculionidae) populations as determined by late-season field disturbance trapping. *Subtropical Plant Science* 60: 58-65, ISSN 1009-7791.

Showler, A.T. (2008b). Longevity and egg development of adult female boll weevils fed exclusively on different parts and stages of cotton fruiting bodies. *Entomologia Experimentalis et Applicata* 127: 125-132, ISSN 0013-8703.

Showler, A.T. & Cantú, R.V. (2008). Effect of adult boll weevil feeding on cotton squares. In: *Proceedings of the Beltwide Cotton Conferences*, pp. 1419-1421. National Cotton Council, Memphis, Tennessee, USA, ISSN 0032-0889.

Showler, A.T. & Robinson, J.R.C. (2008). Cotton harvest at 40% versus 75% boll-splitting on yield and economic return under standard and proactive boll weevil (Coleoptera: Curculionidae) spray regimes. *Journal of Economic Entomology* 101: 1600-1605, ISSN 0022-0493.

Showler, A.T. (2009a). Free amino acid profiles in reproductive and rind portions of cotton fruiting bodies. *Subtropical Plant Science* 61: 37-48, ISSN 1009-7791.

Showler, A.T. (2009b). Roles of host plants in boll weevil range expansion beyond tropical Mesoamerica. *American Entomologist* 55: 228-236, ISSN 1046-2821.

Showler, A.T. (2009c). Three boll weevil diapause myths in perspective. *American Entomologist* 55: 42-50, ISSN 1046-2821.

Showler, A.T. (2010). Do boll weevils really diapause? *American Entomologist* 56: 100-105, ISSN 1046-2821.

Steedman, A. (1988). *The Locust Handbook*. Overseas Development Natural Resources Institute, ISBN 0859542815, London, England.

Sterling, W.L. & Adkisson, P.L. (1966). Differences in the diapause response of boll weevils from the High Plains and Central Texas and the significance of this phenomenon in revising present fall insecticidal control programs. *Texas Agricultural Experiment Station Bulletin 1047,* College Station, Texas, USA.

Stoner, A. (1968). *Sphaeralcea* spp. as hosts of the boll weevil in Arizona. *Journal of Economic Entomology* 61: 1100-1102, ISSN 0022-0493.

Sturm, M.M. & Sterling, W.L. (1990). Geographical patterns of boll weevil mortality: observations and hypotheses. *Environmental Entomology* 19: 59-65, ISSN 0046-225X.

Summy, K.R.; Cate, J.R. & Hart, W.G. (1988). Overwintering strategies of boll weevil in southern Texas: reproduction on cultivated cotton. *Southwestern Entomologist* 13: 159-164, ISSN 0147-1724.

Texas Boll Weevil Eradication Foundation (2011). *Texas Boll Weevil Eradication Foundation, Inc.* http://www.txbollweevil.org/.

Texas Department of Agriculture (2002). Administrative Code, Title 4, Part 1, Chapter 20, Subchapter A, Rule 20.1, Texas Department of Agriculture, Austin, Texas, USA.

USDA-APHIS (United States Department of Agriculture-Animal and Plant Health Inspection Service). (2007). *Boll Weevil Eradication*. USDA-APHIS, Washington, D.C., USA.

Vigueras, A.L. & Portillo, L. (2001). Uses of *Opuntia* species and the potential impact of *Cactoblastis cactorum* (Lepidoptera: Pyralidae) in Mexico. *Florida Entomologi*st 84: 493-498.

Waibel, C.W. (1953). Varieties and strains of citrus originating in the Lower Rio Grande Valley of Texas, pp. 18-24. In: *Proceedings of the Seventh Annual Rio Grande Valley Horticultural Institute*, G.P. Wene, pp. 18-24. Weslaco, Texas, Jan. 21, 1953, USA (no ISSN).

Watson, T.F.; Bergman, D. & Palumbo, J. (1986). Effect of temperature and food on developmental time of the boll weevil in Arizona. *Southwestern Entomologist* 11: 243-248, ISSN 0147-1724.

21

Management of Tsetse Fly Using Insecticides in Northern Botswana

C. N. Kurugundla[1], P. M. Kgori[2] and N. Moleele[3]
[1]*Water Affairs, Private Bag 002, Maun*
[2]*Animal Health, Box 14, Maun,*
[3]*Biokavango Project, Okavango Research Institute, Maun, Botswana*

1. Introduction

The tsetse fly (*Glossina* spp.) is only found in Africa and carries trypanosomes (the disease agents causing human sleeping sickness and animal trypanosomosis- *Nagana*), reaching their southern limits (particularly the *morsitans* group) in Botswana and Kwazulu Natal in South Africa. The tsetse fly transmitted disease is a serious problem in Sub-Saharan Africa and it is estimated that the removal of this disease could double livestock production and markedly increase cultivation levels. It is estimated that potential distribution of tsetse fly in Africa is 300,000 km^2 (Mathiessen & douthwaite, 1985).

Generally, the economic and social impacts of *nagana* and sleeping sickness on animal production and human health are severe, estimates put annual cattle production losses at US$2.7 billion and >55, 000 people dying from sleeping sickness annually (Budd, 1999). The disease is usually of chronic and debilitating form. It is essentially a problem in rural areas, where the threat and burden of such is a significant contributor to rural poverty and malnutrition.

In Botswana, the tsetse infested area covered ≤ 5% but has had significant impact on livestock and human populations, particularly in Ngamiland and Chobe regions, largely because of the wet areas in an otherwise dry country. Prior to the rinderpest pandemic of 1896 which significantly reduced tsetse populations in much of east and southern Africa as a result of the critical loss of food source, the distribution of tsetse fly in Botswana had reached its historical limits of approximately >20,000 km^2 (Ford, 1971; Jordon, 1986). As the tsetse populations recovered from the rinderpest epizootic the incidence of trypanosomosis increased. For instance, between 1949 and 1960, the cattle populations in Chobe District declined by ≤ 95% (Lambrecht, 1972). Ploughing along the flood plains is a common local practice in these areas, and whilst beneficial in the sense of optimal exploitation of soil moisture, this practice carries the risk of potential exposure of farm workers to tsetse fly bites. An average of 50 (geometric mean range = 13-272) trypanosomosis cases was recorded between 1957 and 1977 in Maun Hospital each year. Today the Okavango Delta, Kwando-Linyanti-Chobe areas are the most important destinations for international tourists and one of the major sources of revenue for the country. The risk of sleeping sickness within these areas is perceived as potential threat to the tourism industry, both locally and nationally (RTTCP, 1995).

However, at the end of the rinderpest epidemic, a rebound of the tsetse population in northern Botswana and the subsequent disease challenges led to the introduction of intensified control efforts in the mid-twentieth century using conventional and improved insecticide based methods. This chapter therefore reviews the history of management of tsetse fly control in Botswana, with specific focus on the factors that influence tsetse fly distribution in Botswana, methods of insecticide applications to control tsetse fly, effectiveness of the control methods, and monitored environmental impacts.

2. History, distribution, control and management of tsetse fly in Botswana

Comprehensive historical review of tsetse distribution in Botswana up to the 1980s is provided by Davies (1980). Early records largely from European explorers suggest that tsetse occurred in a continuous belt from Angola, through Caprivi Strip (Namibia) and then the Kwando-Linyanti and Chobe River systems, then via the Selinda Spillway to cover most of northern Botswana's Okavango Delta and its immediate surroundings including Nxai Pan N. P (Figure 1). The history of control and management of tsetse in Botswana is detailed below.

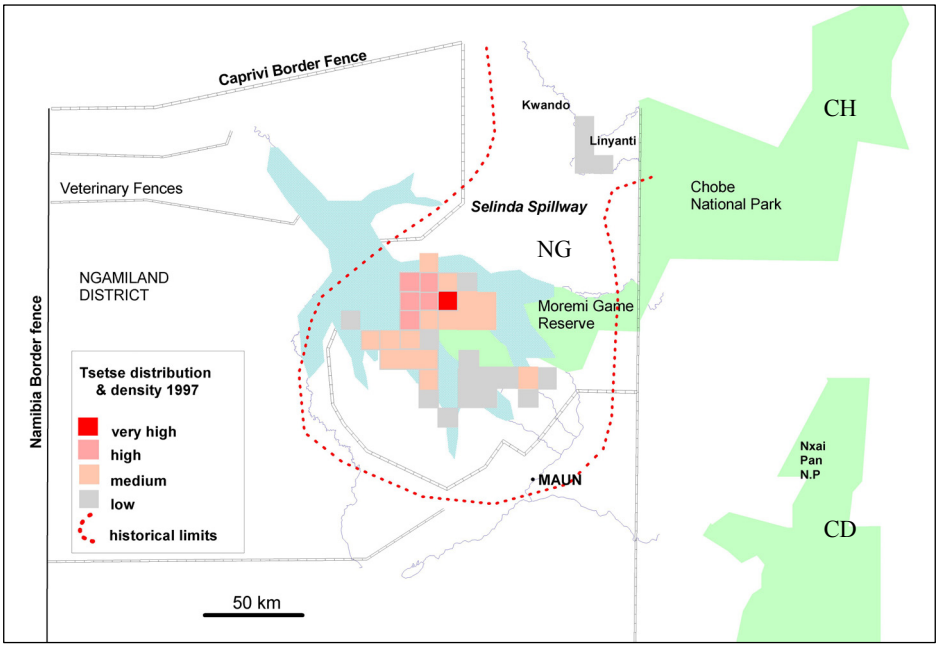

Fig. 1. Distribution of tsetse flies in Botswana (NG = Ngamiland District – Okavango Delta, Maun, Kwando Linyanti; CH = Chobe District – Chobe and Kasane; and CD = Central District – Nxai Pan N. P)

2.1 1940-1960 early methods of tsetse control

The disease Trypanosomosis (sleeping sickness and *Nagana*) is the primary reason for controlling tsetse fly in Botswana. In the early 1940s, cattle *Nagana* in parts of Ngamiland was so bad that a government tsetse control department was formed to combat the disease.

The earliest methods used for reducing tsetse numbers in Botswana (and throughout Africa), were large-scale clearing of bush and vegetation where tsetse fly rested, thus denying them shelter, and elimination of wild game animals which provided the natural food source of the fly. However, both these methods had huge environmental implications and were gradually phased out when alternative methods became available. Nonetheless, bush clearing continued as a complementary method until the mid 1960's (Davis, 1980). Game fencing also emerged as alternative, especially to limit the incursion of game animals into the settled areas of the Okavango Delta fringes.

2.2 1960-1972 residual ground spraying

The method of controlling tsetse using chemicals became common in the 1940s following the discovery of chlorinated hydrocarbon insecticides. In Botswana, residual insecticides such as DDT were introduced in 1967 by applying it to selected tsetse resting sites using knapsack spraying machines. This technique, known as *selective ground spraying*, targeted only about 20% of all potential tsetse resting sites in the woodland, including tree trunks, lower large branches, rot holes and holes on the ground such as ant-bear holes, springhare and hyena dens (Davies, 1980). The residual insecticides would remain available (even after spraying) to tsetse which emerged from underground pupal sites for 2 to 3 months after spray treatment. Given the accessibility challenges within the Okavango Delta, the ground spraying method of control got restricted only to the peripheral areas around the western Delta, the 'Maun Front' or along the Savuti Channel, and it was found to be unsuited to the swamps and island mosaics of the delta interior.

2.3 1970-1990 non-residual aerial spraying

Following preliminary trials carried out in 1971, Botswana's tsetse control strategy switched almost entirely to aerial spraying of insecticide using the Sequential Aerosol Technique (SAT). Very low dosages of endosulfan, and later a cocktail of endosulfan and synthetic pyrethroid insecticide were applied several times over the tsetse habitat to cover all the emerging tsetse pupal period. At best, the early SAT campaign by aerial spraying (Davies, 1979) reduced the tsetse distribution limits from 20,000km^2 to 5,000km^2 but still tsetse could not be eliminated completely despite that being the primary objective from the onset.

2.4 1990-2000 traps and targets

In 1992 Botswana (following other African countries) adopted the use of chemically-impregnated tsetse screens or targets and traps known as the *odour-bait technique* (Vale and Torr, 2004). The system was pioneered in Zimbabwe in the 1980s and became wildly used due to its perceived environmental sensitivity. Targets were treated with synthetic pyrethroid insecticides, particularly deltamethrin suspension concentrate formulation of 0.6% (w/v) following the treatment procedure (Kgori et al., 2006). In the technique the insecticide is applied only to the target screens and not the surrounding vegetation. All traces of the insecticide would therefore disappear once the targets are removed. The concept was well received and particularly suited for the Okavango's pristine wilderness.

Overall, the effectiveness of targets depended on the management and institutional capacity of the government's Tsetse Control Department. Effective distribution of targets and their regular maintenance in the Okavango Delta became a problem as the access was severely impeded by vegetation and terrain. However, a long period of drought ending in 1999 allowed some 25,000

targets to be deployed throughout much of the usually inaccessible parts of the Okavango Delta. These gains were to be reversed when good rains returned in 1999/2000, thus again putting the effectiveness of this tsetse control measure at question. With the above average rainfall, the tsetse fly was able to recover and disperse beyond the confines of the Okavango Delta, taking the threat of trypanosomosis once again back to the people and livestock. Cattle deaths resulting from *Nagana* increased during this period (Sharma et al., 2001).

2.5 2000 onwards - aerial spraying; a reversal in strategy

In the year 2000, the Botswana government initiated a new programme to control tsetse fly and trypanosomosis in and around the Okavango Delta. However, the primary objective remained unchanged; to eliminate tsetse and trypanosomosis from the Okavango Delta and the adjacent Kwando and Linyanti. Tsetse surveys in 2001 showed that the tsetse distribution limits had extended from 5,000 km^2 to about 12,000 km^2. Some 30,000 cattle within the villages surrounding the Okavango delta were clearly at risk of *Nagana* and they were subsequently treated with prophylactic trypanocides.

Reintroduction of aerial spraying became the cornerstone of the new campaign, but this time around targets were used as protective barriers between successive operations to stop tsetse fly from reinvading treated areas. Two aerial spraying operations were planned and executed in succession (in 2001 and 2002) to cover all the infestation in the Okavango Delta (Kgori et al., 2006; Allsopp & Phillemon-Motsu, 2002). In 2001, about 7,000 km^2 (upper box Figure 2) of the northern part of the Delta was aerial sprayed, followed in 2002 by 8,650km^2 (Lower Box Figure 2) of all the remaining infestation in the south (Figure 2). The insecticide of choice was deltamethrin (0.35% (w/v) ulv formulation applied at night using four turbo thrush fixed-wing aircraft. The aircraft were all guided by previously unavailable advanced navigation guidance system (Kgori et al., 2009).

3. Effectiveness of control measures

Apart from limited ground spraying in accessible peripheral areas such as Savuti Channel in the Chobe District, aerial spraying using ulv applications of endosulfan was the only method used to control tsetse fly in Botswana in the 1970s and 80s. Unfortunately for Botswana, since tsetse fly control was conducted largely in aquatic and pristine environment, endosulfan concentrations had to be kept lower than usual; it was applied at maximum of 12 g ha^{-1} compared to operations in other countries where higher levels of 16 to 20 g ha^{-1} were used to achieve comparatively good results (Douthwaite et al., 1981).

Typically, a series of aerial spray treatments were applied at night-time in winter with stable air and temperature inversion conditions in place, and using Piper Aztec or Cessna 310 aircraft from a height of about 15 m above the tree canopy. Under such inversion conditions, the tiny insecticide droplets would drift through the tsetse habitat in order to effectively kill adult tsetse fly. The insecticide was dispersed through a micronair atomizer set to give droplets in the range of 30–40 microns. This ensured that only small quantities of the insecticide were applied to the tsetse habitat. During these early years of aerial spraying, SAT operations were flown without the benefit of advanced aircraft guiding systems which are available today. Therefore spray distribution relied upon far less sophisticated methods of guidance such as ground marker parties with hand held miniflares positioned at each end of the spray run in order to guide the pilots. Also the formulation was non-residual in

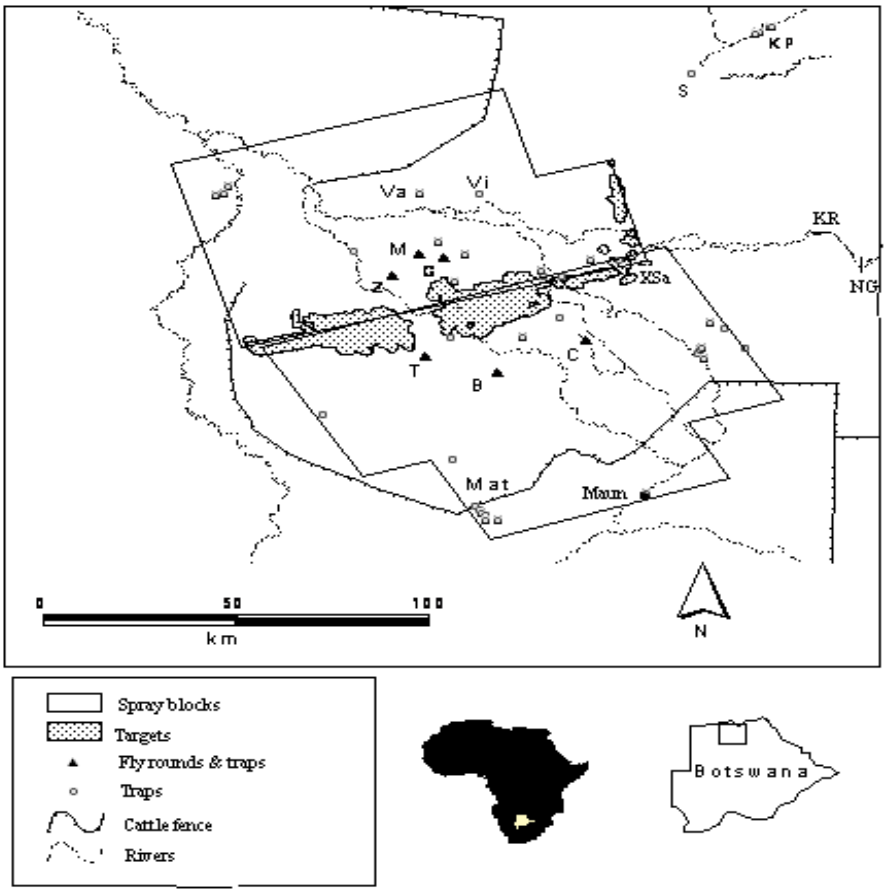

Fig. 2. Operations to control tsetse in the Okavango Delta region of Botswana conducted between 2001 and 2002, and the locations of tsetse and other non-target agents' monitoring sites. Sites indicated by solid triangles indicate the permanent sites at Zimbiri (Z), Mombo (M), Guai (G), Thapagadi (T), Bobo (B) and Chief's Island (C). Open circles indicate occasional monitoring sites used at various times during the spraying operations, including Kings Pool (KP), Selinda (S), Vumbura (Va), Vundumxiki (Vi), and Matsibe (Mat). Source: Kgori et al (2006). Xakanaxa Salvinia monitoring sites (XSa), Khwai River (KR) and North Gate (NR) – Source: Kurugundla and Serumola (2007).

nature; the insecticide deposition remained in the environment only for a short period of time and therefore was non-persistent.

Large-scale trials (1,000 – 4,000 km^2) of aerial spraying with endosulfan in Botswana began in 1973 as a pilot programme to assess the feasibility of even bigger operations to eliminate tsetse fly from the whole of the Okavango Delta (Davies, 1979). By the mid 80s, the scale of the annual operations had increased to about 6,000 – 9,000 km^2 and very good control was achieved. The distribution limit was reduced significantly from 20,000 km^2 to 5,000 km^2 and

the threat of both *Nagana* and human sleeping sickness was effectively controlled due to sequential aerial spraying of insecticide, reduced water levels with less flooded areas and increased drought conditions in Botswana. Sequential spraying was necessary because the tsetse (i.e., young adults) would continue to emerge from the underground pupal stage for several days after each preceding cycle.

However, despite repeated applications complete eradication was not possible. Several possible reasons may have contributed to the failure by early SAT operations to achieve eradication in Botswana - including; low dosages used, lack of boundary protection since odour baited targets were unavailable then, poor navigation and random rather than systematic treatments. The navigation challenges observed often resulted with localized over-spraying which raised environmental concerns, notably fish kill (Merron & Bruton, 1991), but the low dosages used ensured that only limited off-target effects were possible.

4. Deltamethrin

When endosulfan-based tsetse aerial spraying campaign was ended in 1992, targets almost immediately became the next and only preferred option for tsetse fly control in Botswana. At the time, pyrethroid insecticides were also becoming firmly established in the insecticide market and Deltamethrin was the preferred choice for use in the treatment of odour-baited targets (Vale & Torr, 2004). For the next ten years, targets remained the only method used for controlling tsetse in northern Botswana's Okavango Delta, Kwando-Linyanti-Chobe River systems. A new integrated strategy involving the reintroduction of sequential aerial spraying was later introduced in 2001 when targets were proving difficult to effectively implement in Northern Botswana due to accessibility problems.

Prior to commencement of spraying in May 2001, tsetse surveys indicated that tsetse distribution had increased significantly to about 12,000 km^2 from the previous 5,000 km^2 since the end of the last aerial spraying campaign in the 1980s. Increased spread of tsetse fly could be attributed to a long period of no effective control and above average rainfall coupled with increased flood inflows in the Okavango Delta. Also the use of motorized boats as well as airplanes used to transport tourists in and out of the Delta contributed to the spreading of tsetse flies. This was evidenced by the occasional sighting of the tsetse flies in Maun which became a real concern to authorities, and hence the need for concerted effort to reverse the situation.

A two year aerial spraying operation (2001 and 2002, Figure 2) for the north and south of the Okavango Delta using Turbo Thrush Aircraft was planned and implemented in succession to cover the entire Okavango Delta. At the interface of the two spray blocks a target barrier was deployed to prevent tsetse crossing from one unsprayed area to another sprayed area between the successive spray operations. The revised strategy also included a component of the sterile insect technique (SIT) as technical backstop, should SAT fail to eliminate tsetse as was previously the case in the 1980s (Feldmann, 2004).

Improvement in the reintroduction of aerial spraying in 2001, 2002 and 2006 was the availability of latest navigation systems which could ensure precision placement of spraying material and eliminate overdosing or even under-dosing through erratic track guidance. For instance all aircraft were fitted with GPS-guided SATLOC navigation and spray management equipment accurate to about 1m. The system therefore had control on the distribution of the spray application and automatically cut off the spray if the aircraft wondered out of the spray block and indeed the prescribed flight path. The aircraft insecticide dispersal units involved

two boom-mounted micronair AU 4000 rotary atomizers operated at cage speeds of 10,800 rpm and flowrate of 7.6-8.6 l/km^2. Such technology development used in recent SAT operations had positive implications on the distribution and deposition of formulated insecticide droplets and, ultimately on the efficiency and effectiveness of the spray application. During 2001 and 2002 operations, and later in 2006 at Kwando and Linyanti, deltamethrin insecticide (0.35% (w/w) ulv formulation was applied at 0.26 -0.30 g active ingredient (a.i.) ha^{-1}. The higher dose rate of 0.3 g a.i. ha^{-1} was used for the first two cycles when tsetse fly population would normally be at its highest density (Saunders, 1962).

Initially, two successive spray treatments were conducted in 2001 and 2002 to cover approximately 16,000 km^2 in the Okavango Delta (Figure 2). A similar and follow up operation took place in 2006 covering an additional 10,000 km^2 of the Kwando and Linyanti border area, north of the Okavango Delta which also extended across Namibia's eastern Caprivi border into Southern Angola (Figure 3) in order to guarantee complete removal of all the remaining tsetse fly infestation in northern Botswana. This approach ensured that the northern tsetse infestation along the Kwando and Linyanti Rivers did not re-infest the Okavango Delta.

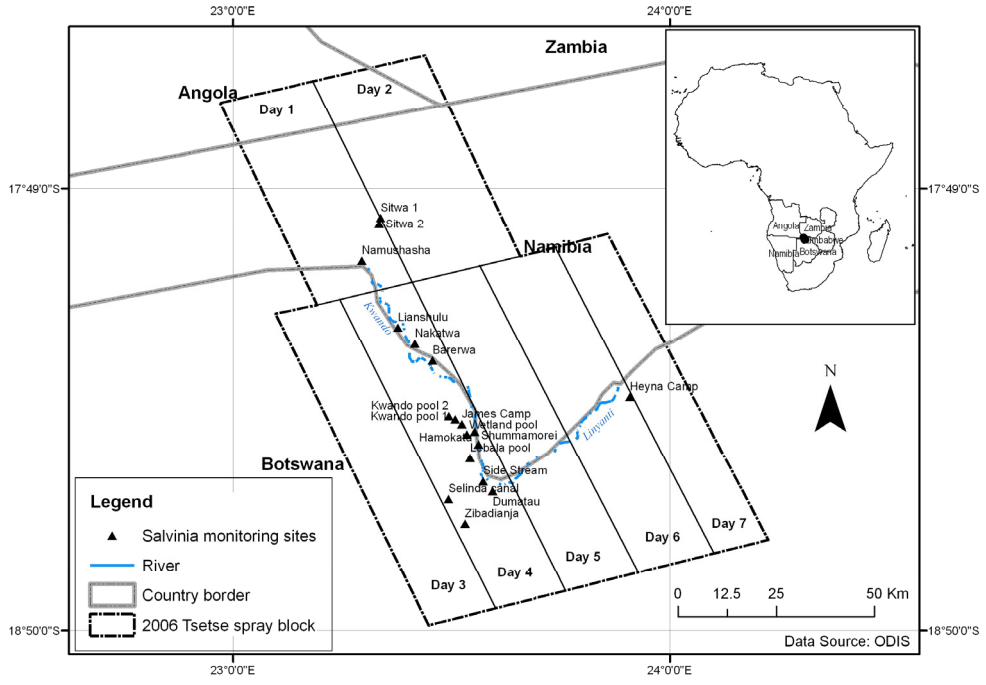

Fig. 3. Area of deltamethrin spraying in 2006. Sites of bioassay experiments and field monitoring areas to assess the impacts of deltamethrin spray on salvinia weevils and other aquatic invertebrates in Kwando-Linyanti River System.

Spraying of the block (7000 km^2) commenced on 3 June 2001. Figure 4 shows the mean daily catch from the five fly-rounds (Figure 4A) and 18 traps within the spray block (4C), and the fly-rounds outside it (Figure 4B). The results from the fly-rounds within the block show that the mean tsetse catch up to the first day of spraying was 44.6 tsetse day^{-1}. Immediately after each cycle the daily catch declined to zero, but then recovered to peaks of 12.8 tsetse day^{-1} after the 1st cycle and 20.6 tsetse day^{-1} after the 2nd cycle. Thereafter recovery was less marked, peaking at 6.3 tsetse day^{-1} after the 3rd cycle, 1.2 tsetse day^{-1} after the 4th cycle and finally no tsetse was found after the 5th cycle. By contrast, the daily catches from fly-rounds outside the spray block (Figure 4B) increased significantly over the period of spray operation.

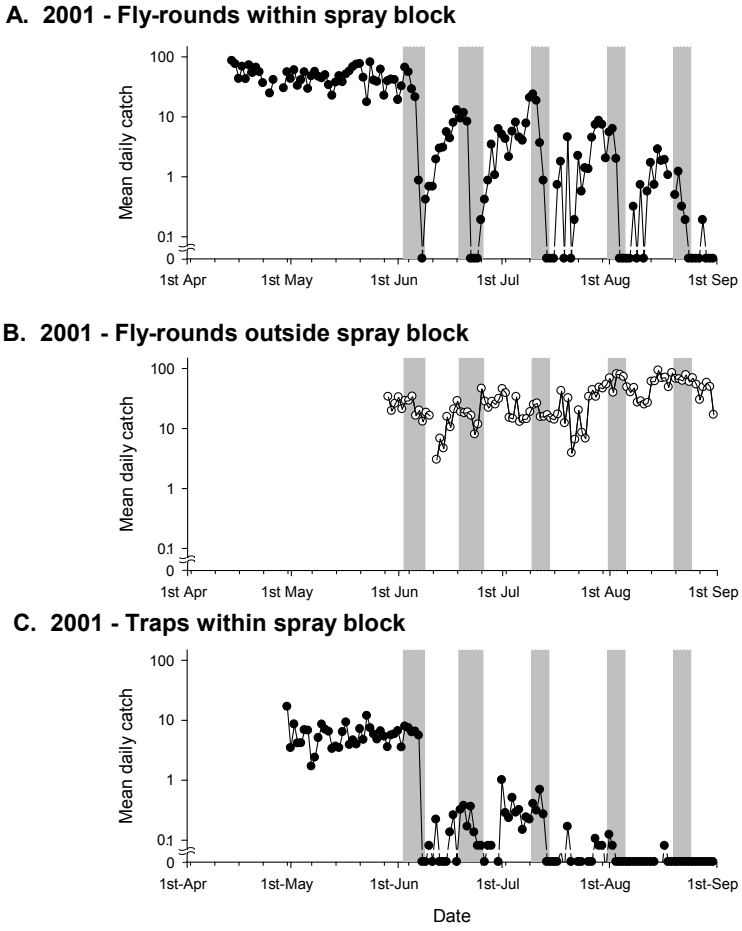

Fig. 4. Detransformed mean daily catch of tsetse from fly rounds and traps within (Mombo, Guai and Zimbiri) or outside (Chief's Island, fly rounds only) the area sprayed during the 2001 control operation. Vertical grey bars indicate the timing of the five spray cycles. Source: Kgori et al., (2006).

Spraying commenced in the south block (8650 km^2) on 16 May 2002. Following the start of spraying, the catches declined dramatically (Figure 5). For instance, the mean daily catch from fly-rounds was 101.7 (±0.026, *n*=148) for the period 10 April - 16 May compared to 0.23 (±0.019, *n*=66) for the period 24 May - 3 June, this being the period between the end of 1st spraying cycle and the beginning of the second cycle (Figure 5A) and after the 4th cycle no tsetse were found. The catches from traps showed a similar decline (Figure 5B). During 2006 campaign, the tsetse surveys started on 13th May 2006 (Day 1 = 13 May 2006. Figure 6 shows that the abundance of tsetse was higher than 100 with fluctuations in Kwando-Linyanti prior to spraying and it reduced to zero after 2nd, 3rd, 4th and zero catches after the 5th spraying cycle (Kgori et al., 2006, Kgori et al., 2009, VEEU-TCD/DAHP 1998).

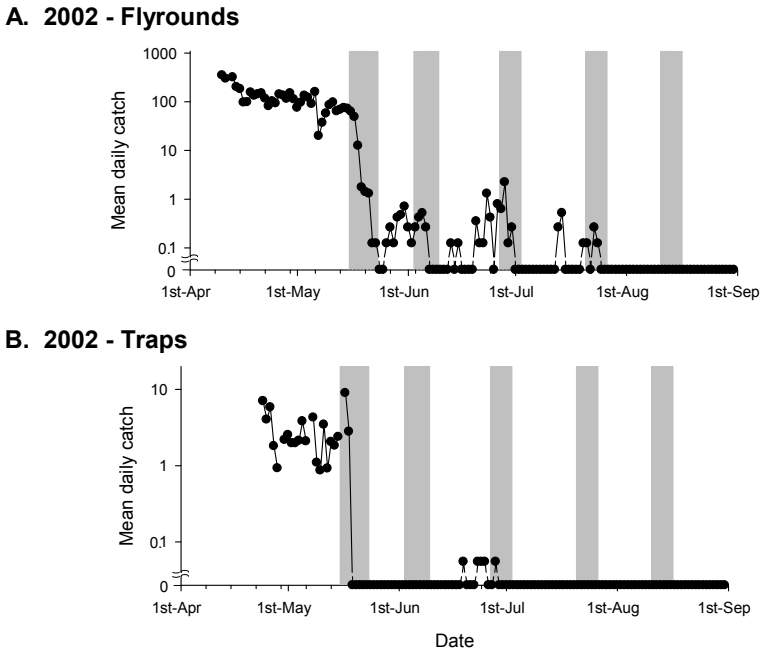

Fig. 5. Detransformed mean daily catch of tsetse from fly rounds and traps within (Bobo Island, Thapaghadi and Chief's Island) the area sprayed during the 2002 control operation. Vertical grey bars indicate the timing of the five spray cycles. Source: Kgori et al., (2006).

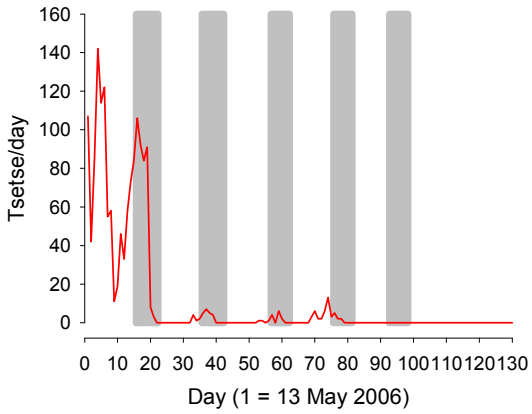

Fig. 6. Tsetse survey results at Kwando/Linyanti 2006 in five spray cycles.

5. Environmental implications

5.1 DDT

'Silent Spring' (Carson, 1962) described the environmental impacts of the indiscriminate spraying of DDT (Dichloro-diphenyl-trichloroethane) in the world and its effects on ecology or human health. It has high potential to bioaccumualte especially in predatory birds and magnify through food; and still highly effective against disease vectors such as malaria parasite (Thomas, 1981). DDT was banned for agricultural use worldwide under the Stockholm Convention but its limited use in disease vector control still continues in some parts of the world to this day and remains controversial (Connel, 1999). Its persistence ranges from 22 days to 30 years depending on the conditions of the ecosystem. It is insoluble in water, less biodegradable and degrades by means of aerobic, anaerobic and photolysis (Thomas, 1981). No specific monitoring of impacts of DDT spraying in Botswana was ever carried out.

5.2 Dieldrin

Deldrin was used world-wide to control locusts and tropical disease vectors, such as tsetse fly and mosquitoes prior to the 1970s, and has persistence of 5-25 years. It has similar characteristics to DDT (Hunter & Robinson, 1968). Botswana was one of the first countries in Africa to monitor the side-effects of chemical control of tsetse fly. In 1964 riverine forests were sprayed with Dieldrin against *G. morsitans* in an area near Maun in the Okavango Delta, Botswana. The effects of the motorized spraying (mist blower) on non-target organisms were observed for a period of 10 days following spraying (Allsopp, 1978). Among the animals found dead were many birds, mammals, reptiles and fish (Graham, 1964). Casual observations of the aerial spray showed no difference between the wildlife populations between the sprayed and unsprayed areas (Davies & Bowles, 1979).

5.3 Endosulfan

Endosulfan degrades more rapidly than DDT and dieldrin; 9% degradation in water between 5-25 days, 15-30 days from vegetation surface, but remains more than 100 days to

one year in soil as residue (Maier-Bode, 1968). It is being phased out globally and a global ban on the manufacture and use of endosulfan was initiated under the Stockholm Convention in April 2011. The ban will take effect in mid 2012 and more than 80 countries became part of the ban (http://en.wikipedia.org/wike/endosulfan). Chemical control methods have been used in anti-tsetse campaigns in at least 20 countries in Africa. Side-effects on non-target organisms have been studied to a greater or lesser extent in only about half of the 20 countries (Table 1).

Country	Year (s)	Type of insecticide + method of application	Reference
Botswana	1964 1975-78 2001-02 2006	Dieldrin - Ground spray (Mist blower) Endosulfan ULV Fixed-wing Deltamethrin – Aerial spray Deltamethrin - Aerial spray	Graham, 1964 Ali, 1978; Fox et al., 1979 Perkins and Ramberg, 2004; 2004a Bonyongo & Mazvimavi, 2007; 2008
Cameroon	1979	Dieldrin – Helicopter	Muller et al., 1980
Chad	1972-1974	DDT- Ground spray	Tibayrence and Gruvel, 1977
Ivory Coat	1979 1968-69	Dieldrin – Ground spray Endosulfan and Decamethrin – Helicopter	Koeman and Pennings, 1970 Everts, 1979
Kenya	1970-72	Dieldrin – Fixed wing	Allsopp, 1978
Namibia	2006	Deltamethrin – Aerial spray	Bonyongo & Mazvimavi, 2007; 2008
Niger	1968 1969-70 1974-76 1977	DDT – Ground spray Dieldrin –Ground spray Dieldrin – ULV Helicopter Endosulfan – Helicopter	Koeman & Pennings, 1970 Koeman et al., 1971 Koeman et al., 1978 Dortland et al., 1977
Nigeria	1975-76 1976 1976 1977-78	Dieldrin/Endosulfan ULV helicopter DDT/Endosulfan – Ground spray Endosulfan – ULV fixed-wing Pyrethroids –Ground and Helicopter spray	Dortland et al., 1977 Koeman et al., 1978 Takken et al., 1976 Smies et al., 1980
Tanzania	1961-71 1979	Dieldrin – Ground spray DNOC (2 methyle-4, 6-dinitro-ortho-oresol), Bush clearing	Sserunjoji & Tjell 1971 Tarimo & Palloti, 1979
Uganda	1963-73	Endosulfan-pyrethroids – Helicopter	Wilson, 1972
Zambia	1968	Endosulfan – ULV fixed-wing	Magadza, 1979
Zimbabwe	1978	Endosulfan – ULV aerial	Cockbill. 1979

Table 1. Anti-tesetse fly campaigns in Africa and impacts studied on the non-target agents.

Deposition

The insecticide deposition in the various habitats in the Okavango Delta is presented in Table 2. However, the range and frequency of individual values are insignificant between them (Douthwaite et al., 1981). Endosulfan concentration in different types of aquatic habitats nine hours after spraying at 9.5 g ha^{-1} varied with depths and the values were insignificant between the various habitats: In marshes ≤ 0.5 m = 0.81 µg l^{-1}; in main river between 1-2 m depth = 1.16 µg l^{-1} and in open pool at ≤ 0.5 m depth = 1.54 µg l^{-1} where as persistence of the insecticide in pools of water declined from 1.42 µg l^{-1} nine hours after spraying and undetectable levels within five days (Douthwaite et al., 1981). Its half life in aquatic environment is between one and five days (Moulton, 1973).

	Open water	Water in grass swamp	Open grassland	Grassland under tree canopy
Mean (Range)	20.7 (1.4-42.9)	17.7 (3.6-45.9)	21.1 (4.5-43.8)	14.3 (2.4-24.5)
Variance	116.1	193.8	188.3	54.2

Table 2. Quantities of endosulfan (µg) drift found on aluminum sheets placed in different habitats and analyzed one hour after spraying 9.5 g ha^{-1} (4 µg per sheet = 1 g ha^{-1}). Source: Douthwaite et al., (1981).

5.3.1 Fish

Bioaccumulation

The major route of uptake in aquatic invertebrates is probably via the digestive system (Roberts, 1975), whereas fish absorb most endosulfan directly from the surrounding water injuring gills, thus reducing the oxygen consumption and disrupting the osmoregulatory function of aquatic organisms (Saravana & Geraldine, 2000). Dortland et al., (1977) have shown that endosulfan applied at 900 g ha^{-1} for tsetse control near West African Rivers produced residue levels of 1.4 and 37.3 µg g^{-1} fish muscle and liver respectively. Experimental applications of endosulafan to fish in paddy fields (Moulton, 1973) showed that gouramis, *Tricho gaster pectoralis* could accumulate the pesticide in abdominal organs to concentrations over 1000 times those in surrounding water. It was found that the absorbed endosulfan in fish was rapidly metabolized to the endosulfan sulphate (Ali, 1978).

The edible fish species tested for bioaccumulation during the endosulfan spraying in Botswana include species of *Clarias, Serranochromis, Schilbe, Haplochromis, Tilapia, Marcusenius*. Endosulfan mean residue concentration with respect to percentage lipid 5 days after the spraying was 0.19 µg g^{-1} wet tissue in muscle, while the maximum found in whole dead small fish was 1.5 µg g^{-1} wet tissue. These values refer to the combined concentration of alpha+beta + endosulfan sulfate - all three compounds are equally toxic to the fish (Anon, 1973). The USA has set a tolerance limit for endosulfan in meat of 0.2 µg g^{-1} fresh weight; this therefore means muscle tissue from living fish in the Okavango would be considered safe (Douthwaite et al., 1981).

Fatty species tend to accumulate the most endosulfan (Douthwaite et al., 1981), and one might perhaps expect these groups would show greater mortality in the field. Such

relationship was however, not apparent in the study because the very fatty insectivore *Marcusenius macrolepidotus* was never found dead after spraying as they accumulated higher residue concentration of 1.002 µg g^{-1} wet weight in its viscera lipid. So the bioaccumulated insecticide can do little damage to vital metabolic processes. Indeed, the laboratory experiments with *Hepsetus* suggest that the fatty lean fish is susceptible and succumb most readily to poisoning to endosulfan and thus fish weight is probably the dominant factor determining the survival, i.e. fry and small individuals are at greater risk. Tests with the predators of fish such as crocodile *Crocodilus niloticus*, fish eagle *Heliaeetus vocifer*, Kingfisher *Ceryle rudis* accumulated endosulfan residues, although these never exceeded 0.2 µg g^{-1} wet weight, except in the visceral fat of the crocodile with 0.783 µg g^{-1} wet weight suggesting again that fatty organs tended to accumulate the highest residue concentrations (Douthwaite et al., 1981).

Mortality

During ultra-low-volume aerial applications of endosulfan at 14g ha^{-1} for control of tsetse fly in savanna woodland, Zimbabwe, no deleterious effect of the insecticide was demonstrated, other than fish (*Tilapia* spp.) in shallow water (Cockbill, 1979). In general, endosulfan killed small fish first, (*Alestes lateralis)* although almost all species were ultimately affected by the 10 g ha^{-1} spray. An endosulfan spray done in the Okavango Delta in 1978 showed that the fish mortality extended over a period of 3 days after spraying in four cycles (Table 3). Fish species affected included *Tilapia, Hemichromis, Haplochromis, Pseudocrenilabrus, Serranochromis, Clarias, Barbus, Schilbe and Hepsetus.* Among them, *Tilapia* was the dominant species affected with 65% poisoning followed by *Pseudocrenilabrus* with 13% (Douthwaite et al., 1981).

Fish mortality per hectare (n = 9 sites)			
Cycle 1	Cycle 2	Cycle 3	Cycle 4
12 g ha^{-1}	12 g ha^{-1}	9 g ha^{-2}	6 g ha^{-2}
542.7 ±339.6 (0-3250)	544.9 ±266.5 (0-2344)	672.1 ±336.5 (0-2531)	2.0 ±1.6 (0-16)

Table 3. Fish mortality resulting from endosulfan spray in Khwai River in the Okavango Delta. (SE ± = sd/$\sqrt{n}$). Figures in the parantheses indicate minimum and maximum mortality (Source: Douthwaite et al., 1981).

The general symptoms of endosulfan or other pesticides' poisoning in fish are epithelial lifting (Bucke et al., 1996, Choudary et al., 2003), Hyperplasia (Munshi et al., 1996), muscle hypertrophy and aedema, liver necrosis (Bucher & Hofer, 1993). Samples of liver and brain were tested from *Hepsetus odoe, Tilapia sparrmanii, T. rendalli and Sarotherodon andersoni* throughout the 1978 spraying and during the ensuing 7 months. Whereas liver samples alone were collected from *Clarias ngamensis and C. gariepinus*. In general endosulfan spray induced liver focal (peripheral) necrosis, brain aedema leading to encephalitis, lining in cephalic tissue during the spray periods as well as 15 days after cycle 6 in the species tested. However, the fish showed healing areas of focal necrosis in liver, absence of aedema in brain in the post spray periods after 7 months. Among the species, *Hepsetus odoe* was tolerant to endosulfan showing only slight change in the fatty liver few days after the spray (Douthwaite et al., 1981).

5.3.2 Aquatic invertebrates

Among the Oligochaeta, Chironomidae, Trichoptera and Ephemeroptera groups the numbers of Chironomid larvae reduced significantly (P≥ 0.001), Oligochaets and Ephemeroptera reduced in abundance while Trichoptera showed fluctuations during the 1978 spray. The decrease in Chironomid larvae in the sprayed lagoon was unexpected as the reported LC50 values for the larvae were considerably higher than the residue levels recorded in the study indicating a normal seasonal change. The Ephemeroptera, Oligochaeta and Chironomidae remained almost constant in pre spray, mid spray while in post spray they increased significantly reflecting positive response to the falling water levels rather than related to the endosulfan effects. With the exception of *Hexarthra* sp. the species namely *Filina, Brachionus, Keratella* and *Polyarthra* perecentage representation was considerably lower in the lagoons following the spraying season (Douthwaite et al., 1981).

5.3.3 Terrestrial invertebrates

The abundance of terrestrial invertebrates in floodplains, grassland and in dry land under tree canopies, *Colophospermamum mopane* canopies in the sprayed and unsprayed areas was determined in the Okavango Delta. The major groups in the studies included Chironomidae, Cicadellidae, Diptera, Hymenoptera, Orthoptera, Araneae, Formicidae, Coleoptera. Only in the case of adult *Chironomids* was there a large reduction in numbers in the sprayed site. However their abundance was almost identical in the post spray periods with the 'control sample' attributing doubts over the spray effects. Three of the major groups namely Formicidae, Tenebrionidae and Diptera showed reduction in activity and the most affected genus was the *Pheidole* in Formicidae following the spray. There was a significant decrease in numbers (P≥ 0.05) of spiders in the sprayed site which correspond to the significant increase (P≥ 0.001) in the sprayed site in the 3rd cycle (Douthwaite et al., 1981).

5.3.4 Flying insects

The behaviour of nocturnal insects during the spraying in the Okavango Delta was caught in the water trays in the grassland along the margins of the permanent swamps before and after each spray application in both sprayed and unsprayed areas. In the first spray cycle a sharp peak in mortality was observed in the adult *Chironomid* insects trapped in the sprayed site. However similar results were not obtained during subsequent spray cycles. This could be due to the emergence of adults from the larvae as the adults have short life-span. Among other groups, only Culicidae, some *Nematocera* and *Diptera spp.* were abundant in water traps and fluctuated in numbers from night to night in both sprayed and control sites, thus showing that there was no consistent evidence for either decreases or increases following each spray cycle (Douthwaite et al., 1981).

5.3.5 Birds

Endosulfan is neither toxic nor cumulative in birds and therefore unlikely to be lethal to them. However, there could be indirect effects on the insectivorous and piscivorous birds through disruption of their food supplies. The occurrence of birds in *Acacia* woodland was monitored before and after spraying in 1978 using transects. Besides similarities in the species in four transects, each transect lost and gained some species between sampling

periods, but no species disappeared from both sprayed and unsprayed transects. A comparison of 39 species was grouped by diet, and by site and method of feeding. To determine the source of the heterogeneity comparisons were made within groups of insectivores, frugivores and granivores and birds with mixed diets. Significant heterogeneity occurred only in the granivores such as *Lamprotornis* starlings that increased in the unsprayed transects but declined in the sprayed transects, and the Grey Hornbill *Tockus nasutus* and Sengegal Coucal *Centropus senegalensis*, which did the opposite. The other insectivore species showed divergent occurrences include Meyer's Parrot *Poicephalus meyeri,* a granivore, Brubru *Nilaus afer* and Red-billed Wood-hoopoe *Phoeniculus purpureus*. These changes in occurrence could, in view of the varied habitats of the species concerned, be as well explained by rainfall, vegetation re-growth between sprayed and unsprayed transects as by the effect of spraying.

The major diet for kingfisher (*Ceryle rudis*) is fish ranging from 28-112 mm and 0.2 to19.1 g in weight. The diet consists mostly of *Tilapia, Haplochromis, Barbus and Pseudocrenilabrus.* These species accumulated the endosulfan in liver and brain and the feeding of these fish by kingfisher may have some concentrations of endosulfan. The study showed the total concentration of alpha and beta endosulfan and endosulfan sulfate in the pooled samples from three kingfisher birds as 0.012 $\mu g\ g^{-1}$ wet weight in the liver and 0.205 $\mu g\ g^{-1}$ weight in the brain. These concentrations are no higher than the levels found in fish and agree with earlier observations (Maier-Bode, 1968).

5.4 Deltamethrin

Deltamethrin is a broad spectrum insecticide, relatively stable but less persistent in the environment than the organochlorine pesticides (Grant & Crick, 1987). It has been in wide use in various crops, in gardens, indoors, outdoors for controlling pests such as mites, ants, weevils, beetles, leafhoppers the world over (Tomlin, 2006). Its persistence ranges from 8 to 48 hours (Erstfield, 1999) in water and 5.7 to 209 days (EFASP, 1999) in terrestrial habitats. Reported concentrations in water are rarely greater than 20 $ng\ l^{-1}$ (Amweg et al., 2006) and were only found to be toxic to honey bees (*Apis* sp.) (Tomlin, 2006). The 2001 deltamethrin spraying over the upper Okavango Delta was done with very little systematic monitoring of the impacts/effects on the non-target organisms (Perkins & Ramberg, 2004). However, the subsequent spraying in 2002 period was done with adequate regular monitoring from the 1st to the 5th cycle. The analysis of data has been assessed by two methods.

i. Sampling was carried out just before each cycle spraying event and in the following day after the spraying. This assumed that significant changes in abundance of individuals in any taxa are likely to be caused by the spraying.
ii. Analysis of trends in numbers of any taxa is compared from cycle 1 to 5 to show the tolerance and susceptibility.

In 2002, the spray deposition as determined by rotating slides was between 23 and 867 drops cm^{-2} and varied considerably within and between sites (Wolski & Huntsman-Mapila, 2002), which could be due to habitat variation, wind, temperature and distance from the flight lines. The wool strands that were exposed to the spraying absorbed the insecticide and revealed that for tsetse fly lethal concentrations were likely to have lasted for four to five days after the spray event (Perkins & Ramberg, 2004). However deltamethrin deposition determined in water, sediments and soil in 2002 and 2006 yielded almost insignificant results except one sediment sample that had low levels of the insecticide (Bonyongo &

Mazvimavi, 2007). Nevertheless, the insecticide deposition as captured by spreading aluminium foil sheets during the monitoring of salvinia weevils gave between 0.2 μg m^{-2} and 6.9 μg m^{-2} in 2002 and 2006 spraying occasions (Kurugundla & Serumola. 2007; Kurugundla et al., 2010).

5.4.1 Aquatic Invertebrates

Impacts - 2002

It is well known that flowing and still water usually differ in biotype composition and hence aquatic macro invertebrate studies were undertaken in still water of pools and flowing channels in Xakanaxa. The results were compared with those of the control site of Khwai River at North Gate. A total of 695 macro invertebrate samples and 200 zooplankton samples were collected, and 64 macro invertebrate families were identified. In channels, abundance declined by 46% after five spray cycles (927 individuals to 520) and in lagoons it declined by 25% (1230 individuals to 917). However, in the reference control site in Khwai River at North Gate, the abundance increased slightly over five spray cycles (789 individuals to 839) (Palmer, 2002).

Samples fixed in 75% ethanol were identified to family in the field using Gerber & Gabrial, (2002) and Davies & Day, (1998). A total of 47 taxa were recorded in channels and 49 taxa in lagoons and in total 65 taxa were identified, of which 23 taxa commonly occurred consistently in samples before and after the spraying. Out of these common taxa 6 showed distinct rapid declines after the first spray cycle and had disappeared completely after the fifth cycle. This corresponds to a loss of 26% of common taxa and was likely caused by the deltamethrin spray deposition. Whereas at North Gate on the Khwai River control site, the number of taxa increased from 29 to 30 after five spray cycles.

It has been shown that the mortality differs between the channels and lagoons and also between the taxonomic groups. Among 64 taxa, more than 24 have been found to be susceptible while few taxa (11) were found to be resistant to the insecticide spraying. Mollusks are probably more physiologically resistant to spraying than insects for obvious shell protection. The other survivor insect families include Chironomidae (non-biting midges), Ceratopogonidae (biting midges), Libellulidae (hairy dragon flies) and Caenidae (crawling mayflies). All these entire insect families live in the sediment, which may function as a protection against the insecticide spray, which is less bioavailable as the pyrethroids could be partitioned and adsorbed to various organic sediments (Muir et al. 1985). On the other hand, the elimination rate recorded for the Hemiptera (water bugs) Carduliidae (dragonflies), for most Ephemeroptera (mayflies) families and some other families could be understood by their active behavior in free water surfaces, sediment and vegetation surfaces. In particular the air breathing behaviour exhibited by most Hemiptera and Coleoptera (beetles) force them to come into contact with deltamethrin and oil-based carrier, paraffin that accumulate on water surface as a thin film (Perkins & Ramberg, 2004; Bonyongo & Mazvimavi, 2007).

Recovery - 2003

The recovery monitoring was measured in 2003 at the same periods and similar sites to those used in 2002 in order to assess recovery at community, family and morphospecies levels. The total abundance after a year was still significantly lower: 39% in channels and

60% in lagoons. The spraying caused significant reductions in abundance of many sensitive taxa often specific for respective habitats like lagoons and channels. What remained in both habitats was a common group of resistant species. The distinct feature is that the reappearance of some of the sensitive taxa that was affected in 2002 spraying was found again during the 2003 recovery studies. At the family level, the most negatively affected families were Atyidae (shrimps) which are characteristic of areas of permanent flow, and Pleidae (pygmy backswimmers), that are found in more seasonal areas. The abundance of Naucoridae (creeping water bugs) was significantly lower than the benchmark as well. Out of 39 identified morphospecies four (10%) were classified as sensitive as they did not reappear during the 2003 recovery study. Three species belonged to the family Notonectidae (Backswimmers) and one to Naucoridae (creeping water bugs), which might reflect a loss from the system. It is however difficult to assign this to persistent post-spray impacts of deltamethrin as the natural variation and abundance in aquatic invertebrates from year to year in the Delta is unknown (Palmer & Davies-Coleman, 2003)

Impact – 2006

Samples were collected at 21 sites in the main Kwando- Linyanti Rivers, Zibadianja and Dumatau lagoons, floodplains and on the vegetation of *Phragmites*, papyrus (*Cyperus papyrus*), hippo grass (*Vossia cuspidata*) and water lilies, (*Nymphaea* spp.) using kick nets (Picker et al 2002). The abundance of macro-invertebrates reduced between 10 and 90% in all the sample sites and affected marginally in the rivers, lagoons and the floodplains. About 70% macro-invertebrates were reduced in numbers from the vegetation types. Abundance of the invertebrate taxa reduced between 40 and 90% except *Chironomids* which appeared to increase during the spraying period. Twenty-four families of macro invertebrates were knocked down almost completely when compared between pre-spray and at the end of spray. About 11 of the total families survived the five spraying cycles (Masundire & Mosepele, 2006).

Recovery – 2007

An average of about 25% of the macroinvertebrates in 24 taxa (Table 4) that were present before spraying did not reappear one year after spraying. Perkins & Ramberg (2004a) reported 26% loss in taxa following spraying in the Okavango Delta in 2002. The mean abundance of all macro-invertebrates was only 11% in Kwando River while more than 100% in Linyanti River, Floodplains, Zibadianja and Dumatau lagoon areas. The *Phragmites* – and hippo grass dominated habitats had still below 50% of pre-spray levels of abundance and in other sites the invertebrates recovered well. Families such as Atydae and Dytiscidae were still well below pre-spray levels while Corixidae, Hirudinea and Chironomidae exceeded their pre spray levels of abundance. Planorbinae appear to have been little affected by the spraying (Masundire, 2007).

5.4.2 Terrestrial invertebrates

Impact – 2002 and 2006

In 2002, sampling was done by collecting the terrestrial invertebrates that had been knocked down by the aerial spray, under tree crowns of *Kigelia Africana, Lonchocarpus capassa* and *Combretum imberbe* on plastic sheets with an area of 3 m^2 in the riparian zone and under *Colophospermum mopane* in the drier land (Dangerfield, 2002). In 2006, similar studies were

conducted in Kwando during the spraying in the woodland dominated by *Croton megalobotrys, Colophospermum mopane, Combretum imberbe, Lonchocarpus nelsii* and in open vegetation dominated by *Pechuel-loeshea leubnitziae* and *Cynodon dactylon* (riparian grass land).The results were compared to the control sites in unsprayed Khwai River at North Gate. Percent declines in terrestrial invertebrate abundance in combined woodland types in 2002 (Dangerfield, 2002) and 2006 were 64% and 63% respectively. All other common taxa showed significant declines as well with the exception of Flies (Diptera Families, Chironomidae and Ceratopogonidae) that have their larval stages in water and therefore produced mass swarming during spring and early summer.

During 2006, 21 taxa groups belonging to 14 families were identified to species level compared to 31 families of flies identified to morphospecies during 2002 spraying. There was marked decrease (50%) in arthropod species richness in the spray block among the woodlands as compared to pre-spray and after spray cycle 5. Significant difference in the composition of families of flies was recorded between the 2002 and 2006 monitoring. The most common families of flies during the 2002 and 2006 monitoring were Tabanidae, Tipulidae, Muscidae, Calliphoridae, Anthomyiidae, Syrphidae, Tephritidae. Spider families sampled in 2002 and 2006 were the same with the exception of family Theraphosidae which was first recorded during 2006. Family Heteropodidae was recorded in 2006 but not in 2002. Family Oxyopidae disappeared after 5th cycle of spraying in both 2002 and 2006. The crickets, particularly *Gryllus bimaculatus* was lost after the 1st cycle and was not captured throughout the remaining spraying cycles. Grasshoppers, *Lithdiopsis carinatus, Truxaloides* sp., *Ailopus thalassinus* and *Eucoptacara exguae* disappeared from the riparian grasslands (Chikwenhere & Shereni, 2006)

Common name	Taxa order	% Reduction – 2002	% Reduction – 2006
Ants	Hymenoptera	11	74
Flies	Diptera	41	60
Mosquitoes	Diptera	71	80
Beetles	Coleoptera	84	43
Wasps	Hymenoptera	23	65
Leaf hoppers & bugs	Hemiptera	46	45
Spiders	Arachnida	30	24

Table 4. Effects of deltamethrin on terrestrial arthropods dwelling in woodland types after spray cycle 2 in 2002 and 2006 (Source: Bonyongo and Mazvimavi, 2007).

In 2006, mosquitoes suffered the highest reduction of 80% followed by ants with 74%, the wasps had 65% reduction, while flies reduced by 60%. The rest of the groups listed declined below 50% and the least reduced was spiders at 24%. Conversely, ants were the least abundant while maximum reductions were found in beetles at 84% followed by mosquitoes with 71% reduction in 2002 (Table 4).

Recovery - 2003 and 2007

In May and August 2003 and 2007 sampling of terrestrial invertebrates in the selected woodland tree types yielded interesting results in the Okavango and Kwando respectively. In the key insect groups, the spider abundance recovered in all cases to pre-spray levels; beetle abundance had not recovered on *K. africana*, but recovered on other tree species; there

was no change in fly or ant abundance between 2002 and 2003 while Hemeptera abundance was significantly greater in 2003 than before the spray events. Fifty-seven morphospecies were found for the first time in 2003 indicating that the species that were lost from 2002 had started to recover in 2003. This shows that there is great variation between years in the composition of invertebrates (Dangerfield & McCulloch, 2003). There was sharp increase in the flies and mosquitoes in Kwando/Linyanti areas in the recovery periods of 2007. Beetle abundance was highest at above 60% and wasps and ants increased more than 100% a year after the spraying. However crickets, *Gryllus bimaculatus* were not detected in 2007 where as Grasshoppers *Lithdiopsis carinatus, Truxaloides sp., Ailopus thalassinus* and *Eucoptacara exguae* that had disappeared from the riparian grasslands started to recover in 2007 (Chikwenhere, 2007)

5.4.3 Flying insects

About 7,500 individuals, mostly flies, were sampled in 2002. Within each cycle catches were lower in the days after the spray event but increased between the spray cycles and much greater in the subsequent pre-cycle catches. Increase in the abundance of flies over the cycles was most likely due to increasing temperatures and the arrival of the annual flood in the sampled areas.

5.4.4 Fish

Using gill nets, the abundance of fish in the Kwando and Linyanti Rivers were studied. In general, a comparison of relative abundance before and after the five spray cycles remained stable. Relative abundance in fish in a given water body reflects the seasonal events, water levels, feeding and breeding behaviour (Mosepele, 2006). However there was decrease in species diversity from the pre spray period until cycle 2 and diversity remained stable after cycle 5. Being an opportunistic predator (Teferra et al., 2003, Mosepele et al., 2005) slight decrease in *Schilbe intermedius* is caused by feeding of knocked down terrestrial and aquatic invertebrates. Increased competition for food between the species does occur. *Hepsetus odoe* and *S. intermedius* were pscivorous predators (Merron & Bruton, 1991) and deltamethrin would have increased inter-specific competition for food between these two species as the feeding rate of *S. intermedius* progressively decreased from the pre spray periods until cycle 5. *Brycinus lateralis* and *B. poechii* feed on small aquatic invertebrates and therefore it was possible that their abundance decreased in the river systems due to decreased food supply caused by deltamethrin spray. The lack of significant change in the feeding behavior of *Marcusenius macrolepidotus* during the spraying suggests that its dominant prey item (Chironomids) were not significantly affected by deltamethrin.

5.4.5 Birds

Using circular point counts for monitoring forest birds, transects for *Acacia* thorn-veld species, and boat surveys for water dependent species, sampling was carried out on three occasions in the Okavango Delta. In summary, there were some local changes in bird populations during the spraying, but these changes could not be attributed to effects of deltamethrin (Pendleton, 2002; Perkins & Ramberg, 2004). The spraying of deltamethrin over the Kwando-Linyanti areas in 2006 did not have discernible negative effects on any of the bird species monitored (Slaty Egret, Arnot's Chat, raptors and

vultures). The only significant effect recorded was increased feeding success of Slaty Egrets during spraying due to the negative effect of the deltamethrin on its prey. This was short-lived and the temporarily depressed fish stocks did not cause the Slaty Egrets to vacate the area.

6. Aquatic plants (*Salvinia molesta*) and biological control weevil *Cyrtobagous salviniae*

The distribution of salvinia and weevil populations varies widely under field conditions. Therefore, using field and static short-term toxicity bioassay methods (Reish & Cshida, 1987) in the Okavango (Figure 2) and iron cages representing open conditions in Kwando (Figure 3), the impacts of the insecticide on the weevil were determined during the five sequential aerial spray cycles. The controls were maintained in unsprayed area of Khwai River at North Gate. Assessments of the impacts were carried out at 12, 36 and 60 hours after the spray in each cycle from basins and cages as well as in field conditions before and after each spraying cycle (Kurugundla & Serumola, 2007, Kurugundla et al., 2010).

Mean survival of the 50 adult salvinia weevils extracted using Berlese funnels (Boland & Room, 1983) in the controls was generally in the range of 45.5 (mortality = 09.0%) to 48.4 (mortality = 03.2%) in number. The range of weevils' survival in closed basins exposed to deltamethrin in 2002 was between 25.0 (mortality = 47.0%) and 44.7 (mortality = 04.9%) while it was 33.7 (mortality = 27.2%) and 39.3 (mortality = 16.4%) in cages in 2006 with respect to controls (Table 5). However, in the 4th cycle of 2006 spray, the weevil mortality was 52.5% (live weevils = 22.0 ±1.8) (Table 5), which was obviously due to the formation of ice crystals and the cold conditions in the weevil extraction cups (≤ 50C) increasing the weevil mortality significantly ($P \leq 0.05$), but not necessarily as a result of deltamethrin spray drift. Cesida (1980) found that pyrethroid toxicity increased at low temperatures.

The percentage deltamethrin collected on the aluminium foil sheets was in the range of 0.2% to 6.9% of the applied rate and the insecticide drift is varied depending on the inversion, wind direction and temperature (Perkins & Ramberg, 2002; Bonyongo & Mazvimavi, 2007). Significant difference in weevil mortality was observed between 2.3% and 6.9% (Table 5) deltamethrin deposition and did not show significant mortality at less than 2% deposition as determined from the aluminium foils (Kurugundla & Serumola, 2007; Kurugundla et al., 2010). The fluctuations in the abundance of weevils in the field Salvinia infestations at the sites of Xakanaxa (Figure 2) and Kwando (Figure 3) after praying was due to the spatial distribution of weevils and to less breeding during winter (Forno et al., 1983; Naidu et al., 2000) despite some spray effects. In all five cycles the abundance of weevils in 20 standard plants was higher than one (Forno, 1987) showing that weevils maintained their equilibrium at Shummamori, Hamokata, Lebala and Selinda (Figure 3) during the spray programme.

Although deltamethrin deposition caused significant weevil mortalities, the insecticide did not affect the weevil ability to control salvinia. It might be difficult to relate the declines of weevils in the field to the deltamethrin toxicity, as toxicity is influenced by factors such as temperature, season of spraying, habitat conditions and a protective mechanism possessed by the life stages of weevils (Schlettwein & Giliomee, 1990). In aquatic habitat conditions deltamethrin aerosols could be diluted, partitioned and adsorbed onto various organic sediments (Muir et al., 1985), which would reduce the toxicity not only to the weevils but several aquatic invertebrates. It is also suggested that the vegetation might act as a limiting factor for insecticide deposition on target surfaces, unlike in the open areas. On cold nights

the weevils normally hide in buds, roots and beneath the leaves. They deposit their eggs in buds and underneath leaves, and emerging larvae normally feed inside the rhizome (Forno et al 1983). Therefore adults and larvae would often be protected from contact with the insecticide (Schlettwein & Giliomee, 1990).

	Okavango Delta - Basins – 2002				Kwando-Linyanti - Cages- 2006			
Cycle	Control	12 hours	36 hours	60 hours	Control	12 hours	36 hours	60 hours
1	47.2 ±1.2 (5.6%)	*32.0 ±1.3 (32.3%)	*30.5 ±1.4 (35.4%)	*25.0 ±1.0 (47.0%	47.0 ±1.6 (06.0%)	39.3 ±2.4 (16.4%)	37.4 ±4.5 (20.4)	35.7 ±1.8 (24.0%)
2	48.4 ±0.5 (03.2%)	31.7 ±0.9 (34.5%)	38.2 ±1.0 (21.1%)	38.8 ±1.3 (19.9%)	46.5 ±0.6 (07.0%)	39.1 ±2.5 (16.0%)	*35.2 ±3.3 (24.3%)	*35.8 ±2.0 (23.0%)
3	46.0 ±0.6 (08.0%)	42.3 ±0.6 (08.1%)	33.5 ±0.9 (27.2%)	31.0 ±1.4 (32.6%	45.5 ±0.6 (09.0%)	37.7 ±3.3 (17.2%)	*34.4 1.4 (24.6%)	*35.0 ±1.9 (23.1%)
4	47.0 ±0.6 (06.0%)	37.0 ±1.1 (21.3%)	44.7 ±1.3 (04.9%)	37.0 ±1.4 (21.3%	46.3 ±0.9 (07.1%)	*35.9 ±3.1 (22.5%)	*33.7 ±1.7 (27.2%)	*22.0 ±1.8 (52.5%)
5	45.4 ±0.5 (09.2%)	*30.3 ±0.7 (33.3%)	*30.5 ±0.9 (32.8%)	*32.2 ±1.5 (29.1%)	46.3 ±1.3 (07.4%)	*36.1 ±1.7 (22.0%)	36.7 ±1.8 (20.7%)	*34.3 ±3.1 (26.0%)
	Okavango Delta- mean of 5 cycles				Kwando-Linyanti - Mean of 5 cycles			
	Deltamethrin deposition (% m^{-2})		Weevil mortality (%)		Deltamethrin deposition (% m^{-2})		Weevil mortality (%)	
Average	2.7 ±0.5		26.5 ±1.0		4.1 ±0.8		29.7 ±5.1	

Table 5. Mean survival of 50 weevils (SE = sd$\sqrt{n}$) in the Okavango Delta and Kwando-Linyanti in response to deltamethrin spray deposition (% m^{-2}). Figures in parentheses indicate corrected percent mortality with respect to controls in each cycle. * Probability ≤0.05 with reference to controls.

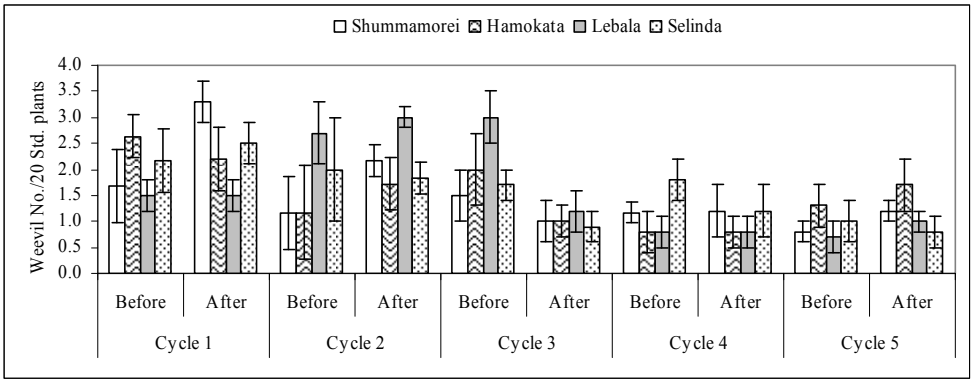

Fig. 7. Number of weevils in 20 plants by standard plant method before and after the spray in five cycles.

Average weevil mortalities are typically 26.5% in 2002 and 29.7% in 2006 at 2.7% and 4.1% deltamethrin respectively (Kurugundla& Serumola 2007; Kurugundla et al., 2010). It was also observed that Paradise pool and Lebala pool were completely covered with the salvinia two months after the end of spraying, yet the weevils controlled the infestation after 7 to 8 months. Aerial spraying of deltamethrin for controlling tsetse fly in any given area is not a continuous process and it is applied only in winter, when the breeding rate of the weevils

generally low. The two important monitoring studies conducted in 2002 and 2006 confirm that, although *C. salviniae* was affected negatively by the aerial spraying of deltamethrin, it recovered thereafter as shown by the subsequent effective control of salvinia in Paradise and Lebala pools in the Okavango delta and Kwando River respectively.

7. Socio-economic implications

No side effects on human health was reported and people expressed their appreciation about the programme. However, there were sporadic reports of irritation to eyes during the spraying as reported by the humans. During the spraying campaign, people continued utilizing crops, fish and wild veld products. No short-term land use changes were observed and no disturbances to domestic live stock (Bendsen et al., 2006). People who moved from the core of tsetse infested areas during 1960s and 1970s have now settled permanently in Caprivi region. The changes in land use did not become apparent during the spraying and in the post spray periods. Botswana is one of the prime wilderness tourism destinations and there were no direct or indirect impacts on the tourism inflow as the result of spraying. The successful eradication of the flies has created an enabling environment for livestock development. No stock losses due to *nagana* have occurred after the spray of 2001, 2002 and 2006 and the carrying capacity of the rangelands has increased. Eighty-two commercial livestock farms, at a size of 2000 ha each, have already been sanctioned by the Namibian Government in the western section of the Spray Block area in Caprivi. Only 8% of the lodges were against the spraying and 92% of the tour operators appreciated the tsetse eradication. The successful eradication of tsetse fly would save the Botswana Government the recurrent costs that were invested annually for the control of flies through the maintenance of 10,000 odour baited targets in the tsetse dominated areas (Bonyongo & Mazvimavi, 2008).

8. Conclusions

The incidence of *nagana* in northern Botswana as a result of tsetse fly spread increased between 1950 and 1960. Besides large scale clearing of bush and vegetation, ground sprays using insecticides such as DDT, Dieldrin, endosulfan and deltamethrin have been used to control the spread of tsetse fly. Application of non-residual spraying of endosulfan in the Okavango Delta, coupled with odour bait technique in the northern wetlands in 1992, reduced the tsetse fly distribution from 20,000 km^2 to 5000 km^2. By exploiting the improved aerial spraying techniques (fitted with GPS-guided spray equipment fixed to the aircraft), Botswana Government sprayed deltamthrin in 2001 and 2002 in the Okavango Delta and in 2006 in the Kwando-Linyanti systems. Almost 10 years following the end of spraying in the Okavango Delta, tsetse fly have still not been found and the threat of cattle trypanosomosis has been quelled. Furthermore, the tsetse frontiers involving the northern tsetse fly distribution along the Kwando and Linyanti Rivers bordering Caprivi region in Namibia – which is part of the continental common tsetse fly-belt has been effectively pushed back into Southern Angola. As such, the threat of reintroduction of tsetse fly back into northern Botswana has been greatly reduced.

Endosulfan aerial spraying did not produce serious harm to terrestrial invertebrates and no significant difference between seasonal and spraying effects was found in aquatic invertebrates at 12 g ha^{-1} of endosulfan applications. Possible exceptions included adult Chironomidae and Hymenoptera other than ants, both of which showed some declines in

the spraying season. Endosulfan had possible influence on migration of fish and *Tilapia rendalli* abundance declines in shallow vegetated areas. Residue of endosulfan was highest in *Schilbe mystus* at 0.04 ppm in muscle and 0.28 ppm in viscera in gram wet weight. The spraying influenced the feeding in king fisher due to behavioural changes. However, physiological studies in fish showed that surviving fish became significantly debilitated although recovery followed cessation of spraying. However, several groups of invertebrates, especially arthropods, are susceptible to the deltamethrin and deltamethrin spraying caused significant reductions in abundance of sensitive aquatic and terrestrial taxa. The results indicate that the surface dwelling arthorpods were affected in great deal rather than the groups such as leaches, snails, pond damsels and others that live in sediments. High elimination rate recorded for the order Hemiptera (water fly), Ephemeroptera (may flies) and Coleoptera (beetles) as they are active in free water and on vegetation surfaces. However deltamethrin did not affect the fish and birds as the result of deltamethrin spraying.

9. References

Ali, S. (1978). Degradation and environmental fate of endosulfan isomers and endosulfan Sulphate in mouse, insect and laboratory model ecosystems. *Diss. Abstr. Int. B* 39: 2117

Allsopp, R. (1978). The effect of Dieldrin, sprayed by aerial application for tsetse control on game animals. *Journal of Applied Ecology* 15: 117–127.

Allsopp R & Phillemon-Motsu, T.K. (2002). Tsetse Control in Botswana – a reversal in strategy. *Pesticide Outlook* 13: (2) 73-76

Amweg, E. L. Weston, D.P., You, J. & Lydy, M. J. (2006). Pyrethroid Insecticides and Sediment Toxicity in Urban Creeks fromCalifornia and Tennessee. *Environ. Sci. Technol.* 40 (5), 1700-1706.

Anon (1973) FAO/Who Evaluation of Endosulfan Residues in Food. FAO/AGP: CP/103, Rome

Bendsen, H., Mmopelwa, G. & Motsholapheko, M. (2006). Socio-economic issues. *In*: Environmental Monitoring of May-August Aerial Spraying of Deltamethrin for Tsetse Fly Eradication in the Kwando-Linyanti and Caprivi Region – 2007. M. C. Bonyongo & D. Mazvimavi (Eds.). HOORC, University of Botswana, pp 224 – 283

Boland, N. P & Room, P.M. (1983). Estimating population density of *Cyrtobagous singularis* Hustache (Coleoptera: Curculionidae) on the floating weed *Salvinia molesta* using Berlese Funnels. *Journal of Australian Entomological Society* 22: 353-354.

Bonyongo, M. C. & Mazvimavi.D (2007). Environmental Monitoring of May-August 2006 Aerial Spraying of Deltamethrin for Tsetse fly Eradication in the Kwando-Linyanti and Caprivi Region. Department of Animal Health and Production. M. C. Bonyongo and D. Mazvimavi (Eds), HOORC, University of Botswana,. 283pp.

Bonyongo, M. C. & Mazvimavi, D. (2008). Recovery Monitoring After 2006 Aerial Spraying of Deltamethrin for Tsetse fly Eradication in the Kwando-Linyanti and Caprivi Region. Department of Animal Health and Production. M. C. Bonyongo and D. Mazvimavi (Eds.)-2008, HOORC, University of Botswana, 105pp

Bucher, F. &Hofer, R. (1993). The effects of treated domestic sewage on three organs (gills, kidney, liver) of brown trout *(Salmo trutta*), *Water Research* 27: 255-261

Bucke, D, Vethaak, D. Lang, T. & Mellargaad, S. (1996). International council for the Exploration of the Sea Techniques in Marine Environmental Science (Copenhagen), Common Diseases and Parasites of Fish in the North Atlantic, Training Guide for Identification

Budd, L. (1999). DFID-Funded Tsetse and Trypanosome Research and Development since 1980. Economic Analysis (vol. 2) Department for International Development, UK.

Carsen, R. (1962). *Silent Spring*. Houghton Tifflin (Eds.), New York, USA.

Cesida, J.E. (1980). Pyrethrum flowers and pyrethroid insecticides. *Environmental Health Perspective* 4: 189-202.

Chikwenhere, C. & Shereni, W. (2006). Terrestrial invertebrates. *In*: Environmental Monitoring of May-August Aerial Spraying of Deltamethrin for Tsetse Fly Eradication in the Kwando-Linyanti and Caprivi Region – 2007. M. C. Bonyongo & D. Mazvimavi (Eds.). HOORC, University of Botswana, pp 63-126

Chikwenhere, C. (2007). Terrestrial invertebrates. *In*: Recovery Monitoring After 2006 Spraying Deltamethrin for Tsetse Fly Eradication in the Kwando-Linyanti and Caprivi Region – 2008. M. C. Bonyongo & D. Mazvimavi (Eds.). HOORC, University of Botswana, pp 15-45

Choudary, N., Sharma, M., Verma, M.P. & Joshi, S.C. (2003). Hepato and neurotoxicity in rat exposed to endosulfan *Journal of Environmental Biology* 24: 305-8

Cockbill, G.F. (1979). Do ultra low volume applications of endosulfan present a serious hazard to the environment? Zimbabwe Rhod. *News Science* 13: 136–137, 139 (TTIQ abstract No. 628)

Connell, D. (1999). *Introduction to Toxicology*, Blackwell Science. p 68.

Dangerfield, J. M. (2002) Monitoring of terrestrial invertebrates. *In:* Environmental Monitoring of Tsetse Fly Spraying Impacts in the Okavango Delta. Perkins JS and Ramberg L (Eds.) -2004. Okavango Report Series No. 2. HOORC, University of Botswana, Botswana. pp 52-110.

Dangerfield, J. M. & McCulloch, K. (2003). Monitoring of terrestrial invertebrate recovery. *In*: Environmental Recovery Monitoring of Tsetse Fly Spraying Impacts in the Okavango Delta Perkins J.S and Ramberg L (Eds.) - 2004. Okavango Report Series No. 3. HOORC, University of Botswana, Botswana. pp 101- 157

Davies J.E. (1979). Evaluation of aerial spraying against tsetse (Glossina morsitans centralis MACHADO) in Botswana. Report No. 209. XLVIIth General Session of the O.I.E Committee. 21-26 May 1979. 40pp. Paris

Davies J.E (1980). A History of Tsetse Control in Botswana – pre 1980. Tsetse Control Division, Maun Botswana. pp 165

Davies, J. E. & Bowles, J. (1979). Effects of large-scale aerial applications of endosulfan on tsetse fly, *Glossina morsitans centralis* Machado, in Botswana. Miscellaneous Report No. 45. Center for Overseas Pest Research, London

Davies B., & J. Day. (1988) *Vanishing Waters.* UCT Press, Rondenbosch Cape Town

Dortland, R. J., Van Elsen, A.C., Koeman, J.H. & Quirijns, J.K. (1977). Observations on side-effects of a helicopter-application of endosulfan against tsetse flies in Niger. Unpublished Report, Department of Toxicology, Wageningen University, Holland

Douthwaite, R.J., Fox, P.J., Matthiessen, P & Russel-Smith, A. (1981). Environmental impact of aerosols of endosulfan, applied for tsetse fly control in the Okavango Delta,

Botswana. Final Report of the Endosulfan Monitoring Project, Overseas Development Administration, London. 141pp

EFASP. (1999). *Environmental Fate Assessment for the Synthetic Pyrethroids*; U.S. Environmental Protection Agency, Office of PesticidePrograms, Environmental Fate and Effects Division, U.S. Government Printing Office: Washington, DC

Erstfeld, K. M. (1999). Environmental fate of synthetic pyrethroids during spray drift and field runoff treatments in aquaticmicrocosms. *Chemosphere* 39: (10), 1737-1769.

Everts, J.W. (ed.). (1979). Side-effects of aerial insectivide applications against tsetse flies near Bouaflé, Ivory Coast. Dept. Toxicology, Agric. Univ., Wageningen, The Netherlands. 115 pp.

Feldmann, U. (2004). The sterile insect technique a component of area-wide integrated pest management of tsetse. In: Maudlin, I, Holems, P and Miles, M (Eds.) The Trypanosomiases. CABI Publising,Wallingford, UK, pp.565-582

Ford, J.U. (1971). The Role of the Trypanosomiasis in African Ecology. A Study of the Tsetse Fly Problem. Clarendon Press, Oxford, UK, 568 pp

Forno I. W. (1987). Biological control of the floating fern *Salvinia molesta* in north-eastern Australia: plant-herbivore interactions. *Bulletin Entomological research* 77: 9-17

Forno I. W., Sands D. P. A., & Sextone, W. (1983). Distribution, biology and host specificity of *Cyrtobagous singularis* Hustache (Coleoptera: Curculionidae) for the biological control of *Salvinia molesta*. *Bulletin of Entomological Research* 73: 85-95

Fox, P.J., Matthiessen, P., & Douthwaite, R.J. (1979). ODM Endosulfan Monitoring Project. Interim Report. Maun, Botswana, 25 pp.

Gerber, A & Gabriel, M. J. M. (2002). Aquatic Invertebrates of South African Rivers. Field Guide WQS, DWAF, Private Bag X313, Pretoria.

Graham, P. (1964). Destruction of birds and other wild life, by dieldrex spraying against tsetse fly in Bechuanaland. *Arnoldia* 10 (1): 1–4.

Grant I. F & Crick , H. O. P. (1987). Environmental impact of sequential applications of deltamethrin aerosols applied for tsetse control in Zimbabwe. London. Tropical Development and Research Institute

Hunter C.G. & Robinson, J. (1968). Aldrin, dieldrin and man. *Food Cosmetics and Toxicology* 6:253-260.

http://en.wikipedia.org/wike/endosulfan. Endosulfan 17pp. Retrieved 20 June 2011

Jordon, A. M. (1986). Trypanosomiasis Control and African rural Development, Longman Group Limited, Harlow, UK, 357 pp

Kgori P. M, Modo, S. & Torr, S. J. (2006). The use of aerial spraying to eliminate tsetse from the Okavango Delta of Botswana. *Acta Tropica* 99: 184-199

Kgori P.M, Orsmond G. and Phillemon-Motsu T.K (2009) Integrating GIS and GPS-assisted navigation systems to enhance the execution of SAT-based tsetse elimination project in the Okavango Delta (Botswana) In Cecchi and Mattioli Edn; *Geospatial datasets and analyses, PAAT T&S Series 9*; 61-67

Koeman, J.H. & Pennings, J. H. (1970) An orientational survey on the side-effects and environmental distribution of insecticides used in Tsetse Control in Africa. *Bull. Environm. Cont. Toxicology* 5: 164–170.

Koeman, J.H., Rijksen, H.D., Smies, M., Na'isa, B.K., & MacLennan, K.J.R. (1971). Faunal changes in a swamp habitat in Nigeria sprayed with insecticide to exterminate *Glossina*. Netherlands. *Journal of Zoology* 21 (4): 434–463.

Koeman, J.H., Boer, W.M.J., Den, A. F., Feith, H. H. Longh, P. C., de Spliethoff, B., Na'isa, K. & Spielberger, U. (1978). Three years observation on side-effects of helicopter applications of insecticides to exterminate *Glossina* species in Nigeria. *Environmental Pollution* 15: 31–59.

Kurugundla C. N & Serumola. O. (2007). Impacts of deltamethrin spray on adults of the gaint salvinia bio-control agent, *Cyrtobagous salviniae*. *Journal of Aquatic Plant Management* 45: 124-129

Kurugundla, C. N., Bonyongo, M.C. & and Serumola, O. (2010). Impact of deltamethrin aerial spray on adult Cyrtobagous salviniae in Botswana. *South Africa Journal of Aquatic Science* 35(3): 259-265

Lambrecht, F. L. (1972). The tsetse fly: a blessing or a cause? In: Farver, M. T. Milton, J. P. (Eds.), The Carless Technology-Ecology and International Development. The Natural History Press, Garden City, New York, USA, pp 726-741.

Magadza, C.H.D. (1969). Aerial application of Thiodan for tsetse eradication-an ecological assessment. A.R.C. of Zambia, unpublished report.

Maier-Bode, H. (1968). Properties, effect, residues and analytics of the insecticide endosulfan, *Residue Reviews* 22: pp 1-44.

Masundire, H. M. & Mosepele, B. (2006). Aquatic invertebrates. *In*: Environmental Monitoring of May-August Aerial Spraying of Deltamethrin for Tsetse Fly Eradication in the Kwando-Linyanti and Caprivi Region – 2007. M. C. Bonyongo & D. Mazvimavi (Eds.). HOORC, University of Botswana, pp 127 – 154

Masundire, H. M. (2007). Aquatic invertebrates. *In*: Recovery Monitoring After 2006 Aerial Spraying of Deltamethrin for Tsetse fly Eradication in the Kwando-Linyanti and Caprivi Region - 2008. Department of Animal Health and Production. M. C. Bonyongo and D. Mazvimavi (Eds.), HOORC, University of Botswana, pp 46-73

Matthiessen, P. & Douthwaite, R. J. (1985). The impact of tsetse fly control campaigns on African wildlife. *Oryx*, 19: 202-209

Merron G.S & Bruton, M. N. (1991). The physiological and toxicological effects of Arial spraying with insecticides on the fish stocks of the Okavango Delta, Botswana. Interim Rep. No4, WWF, 12pp, Gland, Switzerland

Mosepele, K., Williams, L. & Mosepele, B. (2005). assessment of the feeding ecology of the Silver catfish (Schilbe intermedius Ruppal 1832) in a seasonal floodplain of the Okavango Delta, Botswana. *Botswana Notes and Records* 37: 208-217

Mosepele, K. (2006). Fish. *In*: Environmental Monitoring of May-August Aerial Spraying of Deltamethrin for Tsetse Fly Eradication in the Kwando-Linyanti and Caprivi Region – 2007. M. C. Bonyongo & D. Mazvimavi (Eds.). HOORC, University of Botswana, pp 172-195

Moulton, T.P. 1973. The effects of various insecticides (especially thiodan and BHC) on fish in the paddy fields of West Malaysia. *Journal of Malaysian Agriculture* 49: 224-253

Muir D.C.G, Rawn, G. P., Townsend, B. E. & Lockhart, W. L. (1985). Bioconcentration of cypermethrin, deltamethrin, fenvalerate and permethrin by *Chironomus tentans* larvae in sediment and water. *Environmental Toxicological Chemistry* 4: 51-61

Müller, P., Nagel, P. & Flacke, W. (1980). Ecological side effects of Dieldrine application against tsetse flies in Adamaoua, Cameroon. Report presented at Expert Consultation on Envir. Impact of Tsetse Control 9–12 June 1980, Rome.

Munshi, J.S.D, Dutta, H.M., Brusle,J., Gonzalez, I. & Anandon, G. (1996).The structure and function of fish liver in: Fish Biology Munshi, J.S.D., H. M. Dutta (eds.) Science Publishers Inc. New York

Naidu K.C., Muzila, I., Tyolo, I. & Katorah, G. (2000). Biological control of *Salvinia molesta* in some areas of Moremi Game Reserve, Botswana. *African Journal of Aquatic Science* 25:152-155.

Palmer, C. G. (2002). Monitoring of Aquatic invertebrates In: Environmental Monitoring of Tsetse Fly Spraying Impacts in the Okavango Delta-2002, Perkins JS and Ramberg L (Eds.) 2004. Okavango Report Series No. 2. April 2004, HOORC, University of Botswana, Botswana. pp31 – 51

Palmer, C. G & H. D. Davies-Coleman (2003) Recovery monitoring of aquatic invertebrates. In: Environmental Recovery Monitoring of Tsetse Fly Spraying Impacts in the Okavango Delta, Perkins J.S and Ramberg L (Eds.) - 2004. Okavango Report Series No. 3, University of Botswana, Botswana. pp 52-100

Pendleton, F. (2002). Monitoring of birds. In: Environmental Monitoring of Tsetse fly Spraying Impacts in the Okavango Delta, Final Report. Perkins JS and Ramberg L (Eds.) -2004. Okavango Report Series No. 2. HOORC University of Botswana, Botswana. pp 111-130.

Perkins J. S and L. Ramberg L (2004) Environmental Monitoring of Tsetse fly Spraying Impacts in the Okavango Delta-2002. Okavango Report Series No. 2., HOORC,, University of Botswana, Botswana. 150pp

Perkins J. S and L. Ramberg L (2004a) Environmental Recovery Monitoring of Tsetse fly Spraying Impacts in the Okavango Delta-2003. Okavango Report Series No. 3., HOORC,, University of Botswana, Botswana. 157pp

Picker, M., Griffiths, C. & Weaving, A. (2002). Field Guide to insects of South Africa. Struil Publishers. 444pp

Reish D. L. & Cshida, P.S. (1987). Manual methods in aquatic environment research 247, part – 10, Short term static bioassay, FAO Food and Agriculture Organization of the United Nations, Fisheries Technical Paper, Rome 62 pp

Roberts, D. (1975). Differential uptake of endosulfan by the tissue of Mytilus edulis. Bull. Environ. Contam. Toxicol. 13: 170-176

RTTCP (1995). Study of the tsetse fly problem common to Angola, Botswana, Namibia and Zambia. Accounting No. 6 ACP PRP 468. 174-pp. RTTCP, Harare, Zimbabwe

Saravana, B. P. & Geraldine, P. (2000). Histopathology of the hepatopancreas and gills of the prawn Macrobrachium malcolmsonii exposed to endosulfan. *Aquatic Toxicology* 50: 331-339

Saunders, D.S. (1962). Age determination for female tsetse flies and the age composition of samples of G. pallidipes, G.p. fuscipes and G. brevipalpis. *Bulluten of Entomological Research 53: 579-595*

Sharma, S.P, Losho, T., Malau, C., Mangate, K.G., Linchwe, K.B., Amanfu, W. & Motsu, T.K. (2001). The resurgence of trypanosomosis in Botswana. *J.S. Afr.Vet. Assoc.* 72, 232 – 234.

Schlettwein C. H. G. & Giliomee, J.H. (1990). The effects of different dosages of the insecticide mixtures endosulfan/alphamethrin on adults of the biological control agent *Cyrtobagous salviniae* (Coleoptera: Curculionidae) against *Salvinia molesta*. *Madoqua* 17(1): 37-39

Smies, M., Evers, R.H.J., Piejnenburg, F.H.M. & Koeman, J. H. (1980) Environmental aspects of field trials with pyrethroids to eradicate tsetse fly in Nigeria, *Ecotoxicology, Environment and Safe* 4: 114-128

Sserunjoji, S.J.M. & Tjell, T.C. (1971). Insecticide Residues following Tsetse Control in Uganda. Paper No. 13 of a joint FAO/IAEA panel, Vienna, October 1971.

Takken, W., Bruijckere, F. L. G. & Koeman J. H. (1976). Environmental impact studies concerning the fixed-wing aircraft endosulfan applications in the Southern Guinea Savanna zone in Nigeria. Report Dept. Toxicology Agriculture University. Wageningen, the Netherlands.

Tarimo, C.S. & Pallotti, E. (1979). Residues of aerially sprayed DNOC in biological materials. OAU/STRC, 15th meeting ISCTR 1977: 572–578 (TTIQ abstract No. 776).

Teffera, G., Feledi, B. & Motlhabane , D. (2003). The effects of rainfall on the composition and quality of food ingested by two species of fish *Schilbe intermedius* and *Oreochromis massambicus* in Gaborone Dam. *Botswana Notes and Records* 53: 179-186

Tibayrenc, R. & J. Gruvel (1977) La campagne de lutte contre les glossines dans le bassin du Lac Tchad II. Contrôle de l'assainissement glossinaire. Critique technique et financière de l'ensemble de la campagne. Conclusions générales. *Rev. Elev. Méd. Vét. Pays trop*. 30: 31–39.

Thomas D. R. (1981). *DDT: Scientists, Citizens, and Public Policy*. New Jersey: Princeton University Press

Vale, G.A. and Torr, S.J. (2004). Development of bait technology to control tsetse. In The *Trypanosomiases* (Maudlin, I. *et al.,* (Eds.) pp. 533-546, CABI Publishing.

VEEU-TCD/DAHP (1998) Socio-economics of Tsetse Fly Control in Northern Botswana. In collaboration with DFID UK. 92- pp. Gaborone, Botswana

Wilson, Vivian, J. 1972 Observations on the effect of Dieldrin on wildlife during tsetse fly *Glossina morsitans* control operations in Eastern Zambia. *Arnoldia* (Rhodesia) 5: 1-12

Wolski, P and Huntsman-Mapila, P. (2002). Mateorology and spray deposition. *In*: Environmental Monitoring of Tsetse Fly Spraying Impacts in the Okavango Delta-. Perkins J.S and Ramberg L (Eds.) - 2004. Okavango Report Series No. 2. HOORC, University of Botswana, Botswana. pp 17-27

Part 4

Toxicological Profile of Insecticides

22

Trends in Insecticide Resistance in Natural Populations of Malaria Vectors in Burkina Faso, West Africa: 10 Years' Surveys

K. R. Dabiré[1], A. Diabaté[1], M. Namountougou[1], L. Djogbenou[2], C. Wondji[3], F. Chandre[4], F. Simard[5], J-B. Ouédraogo[1], T. Martin[6], M. Weill[7] and T. Baldet[8]

[1]*IRSS/Centre Muraz, BP 390 Bobo-Dioulasso,*
[2]*IRSP/ Ouidah,*
[3]*Liverpool School of Tropical Medicine,*
[4]*LIN/Montpellier UMR MIGEVEC*
[5]*IRD/IRSS, Bobo-Dioulasso UMR MIGEVEC,*
[6]*CIRAD, UR HORTSYS, Montpellier,*
[7]*ISEM, CNRS/Université Montpellier 2, Montpellier,*
[8]*IRD/CIRAD/CREC, Cotonou,*
[1,5]*Burkina Faso*
[2,8]*Bénin*
[3]*UK*
[4,6,7]*France*

1. Introduction

Malaria is a major threat in endemic regions causing at least 1 million deaths each year affecting poor and underserved populations living in tropical and sub-tropical regions. Diseases control ideally entails prevention and treatment of human infections. However, few vaccines are currently available and many pathogens are now resistant to anti-parasitic drugs. Additionally populations from endemic countries have less access to treatments due to economical impediments. Thus, the control of vectors in many instances is the only affordable measure (Beier *et al*., 2008). Mosquito control is mainly achieved by using insecticides and secondarily bio-larvicides (*Bt*-H14, *B. sphaericus*), predators (fish or copepod predators) or parasitic load (fungi), and/or by modifying the physical environment (WHO, 2006). Insecticides target a vital physiological function, leading to mosquito death. Unfortunately, due to their extremely large numbers and short generation span, mosquito populations evolve very rapidly and become resistant to insecticides, leading to repeated field control failures. Resistance results from the selection of mutant individuals able to survive and reproduce in presence of insecticide, the insecticide failing to disrupt the function of its target. In 2007, more than 100 mosquito species were resistant to at least one insecticide, some species being resistant to several compounds (Whalon *et al*., 2008). Very few classes of synthetic insecticides are available today for vector control, the most recent

has been introduced 20 years ago and none are expected in the near future (Nauen, 2007). The low availability of insecticides due to resistance is further reduced in many countries by the removal from the market of compounds for public health because for their toxicity for humans or the lack of specificity in non-target species (Rogan & Chen, 2005).
Resistance is a genetic adaptation to the modification of the environment induced by insecticides. It usually appears locally, sometimes independently in different places, but may spread rapidly through migration (Brogdon *&* McAllister, 1998; Weill *et al*., 2003). However, mosquito resistance is not only due to the insecticides used for mosquito control, but also to the many pesticide pollutions present in their environment which are generated by a large variety of human activities including insect control for agriculture and other house-hold protections. These pollutions may dramatically affect resistance genes dynamics and threaten vector control strategies. The overall pesticides pressure that select resistance in mosquitoes need to be clarified, both in terms of insecticides usage and quantity.
An. gambiae is a complex, with seven sibling species that are closely related and morphologically indistinguishable from each other by routine taxonomic methods (Gillies & Coetzee, 1987). These sibling species are however different with respect to ecological and behavioral characteristics and to vectorial competence. In West Africa, *An. gambiae s.s.* and *An. arabiensis* are the two main species of the complex that transmit malaria, with the former being the most efficient vector due to its high anthropophily (White, 1974, Lemasson *et al*., 1997). Previous study carried out on the species composition in Burkina Faso indicated that *An. gambiae s.l.* was found to be a mixture of *An. gambiae s.s.* and *An. arabiensis* across the Sudan (98.3% *vs.* 1.7%), Sudan-sahelian (78.6% *vs.* 21.4%) and the Sahel (91.5% *vs.* 8.5%) ecotypes (Dabiré *et al*., 2009a). *An. gambiae s.s.* contains two molecular forms, M and S, which co-exist in West Africa (della Torre *et al*., 2005). The M form was predominant in permanent breeding sites such as rice fields, whereas the S form was predominant in temporary habitats notably rain-filled puddles which are productive during the wet season. In Burkina Faso, genes conferring resistance to insecticides display large frequency differences in M and S forms of *An. gambiae s.s.* and *An. arabiensis*. Resistance of *An. gambiae s.l.* to DDT and pyrethroids (PYR) is especially conferred in West Africa by mutation of the sodium channel target site, the L1014F *kdr* (Chandre *et al*., 1999; Diabaté *et al*., 2002; Awolola *et al*., 2005; Nguessan *et al*., 2007). Burkina Faso is composed of three agro-climatic zones and the use of insecticides to control agricultural and human health pests varies considerably in the different zones particularly as the main cotton cropping areas are found in the south west of the country. In this last region, the intensive use of insecticides most notably for fighting the cotton *Gossypium hirsutum* L. pest is thought to have selected insecticide resistance genes in mosquitoes whose breeding sites are exposed to pesticide runoff (Diabaté *et al*., 2002; Dabiré *et al*., 2009a & b). The goal of this chapter is to summarise the resistance to insecticides status mainly in *An. gambiae s.l.* populations throughout these different agro-climatic areas and to discuss how it could limit the efficacy of malaria vector control strategies in short and long terms at the country scale. Such information is vital to determine the suitability of pyrethroids used for bednet impregnation and CX or OP based-combinations for indoor residual spraying (IRS).

2. Materials and methods

In Burkina Faso country-wide surveys associating bioassays and molecular investigations were carried out from 2000 to 2010 through 26 localities and they allow updating the

resistance status to DDT and pyrethroids and the distribution of L1014F *kdr* among *An. gambiae s.l.* into different agro climatic zones (table 1). We were also interested more recently from 2007 to perform bioassays with some insecticides among OP and CX and also to detect the *ace-1*^{R} mutation 2-3 days aged females of *An. gambiae s.l.* issued from wild larvae were exposed to several molecules such as DDT 4%, permethrin 0.75%, deltamethrin 0.05%, bendiocarb 0.1%, chlorpyriphos methyl (CM) 0.04%, carbosulfan 0.04% and fenithrotion 0.04% according to the WHO tube protocol (WHO, 1998). These active molecules were chosen as they represented each family of classic insecticides commonly used in public health. Some of these molecules such as permethrin, deltamethrin and bendiocarb are now in use in Burkina Faso through impregnated bednets and IRS application.

Study sites: Burkina Faso covers three ecological zones, the Sudan savannah zone in the south and west, the arid savannah zone (Sudan-sahelian) which extends throughout much of the central part of the country and the arid land (Sahel) in the north. The northern part of the country experiences a dry season of 6-8 months with less than 500 mm of rainfall per year. Rainfall is heaviest in the south-west (5-6 months) with a relatively short dry season. The varied ecological conditions are reflected in the different agricultural systems practiced throughout the country, from arable to pastoral lands. The western region constitutes the main cotton belt extending to the south where some new cotton areas have been cultivated. All ecological zones support the existence of *Anopheles* species that vector malaria and the disease is widespread throughout the country (Figure 1).

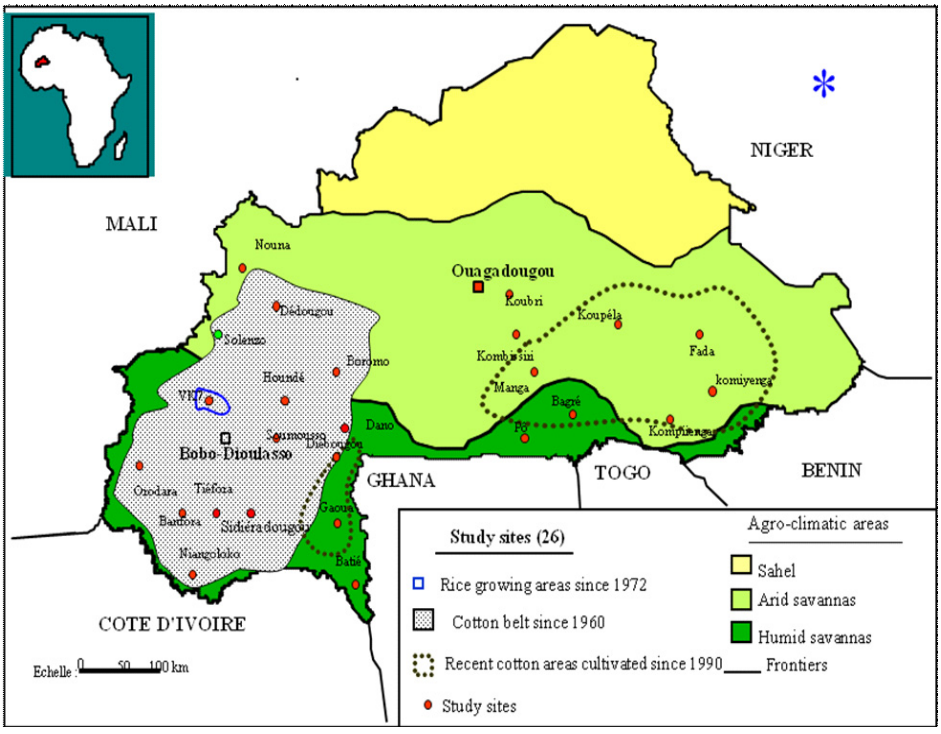

Fig. 1. Localities of the study through the three agro-climatic zones of Burkina Faso.

Study sites	Geographic references	Climatic areas	Environment	Agricultural practices	Recent date of collection
Batié	9°80′N; 2°90′W	Sudanian	rural	cereals, cotton	20/08/09
Gaoua	10°40′N; 3°15′W	Sudanian	sub-urban	cereals, cotton	20/08/09
Diébougou	10°95′N; 3°24′W	Sudanian	sub-urban	cereals, cotton	20/08/09
Dano	11°10′N; 3°05′W	Sudanian	rural	cereals, cotton	20/08/09
Banfora	10°60′N; 4°70′W	Sudanian	sub-urban	cereals, cotton	15/08/09
Sidéradougou	10°60′N; 4°25′W	Sudanian	rural	cereals, cotton	15/08/09
Tiéfora	10°50′N; 4°50′W	Sudanian	rural	cereals, cotton	15/08/09
Orodara	11°00′N; 4°91′W	Sudanian	rural	fruits, cotton	15/08/09
Dioulassoba	11°22′N; 4°30′W	Sudanian	traditionnal-urban	swamp	15/08/09
Soumousso	11°01′N; 4°02′W	Sudanian	rural	cotton	15/08/09
VK7	11°41′N; 4°44′W	Sudanian	rural	rice, cotton	08/08/09
VK5	11°24′N ; 4°23′W	Sudanian	rural	rice	08/08/09
Pô	11°20′N; 1°10′W	Sudanian	sub-urban	cereals, cotton	28/08/09
Houndé	11°50′N; 3°55′W	Sudanian	sub-urban	cotton	10/08/09
Boromo	11°75′N; 2°92′W	Sudan-sahelian	sub-urban	cotton	16/08/09
Solenzo	12°37′N; 3°55′W	Sudan-sahelian	rural	cotton	16/08/09
Dedougou	12°50′N; 3°45′W	Sudan-sahelian	sub-urban	cotton	16/08/09
Nouna	12°70′N; 3°90′W	Sudan-sahelian	sub-urban	cotton	16/08/09
Koubri	12°35′N; 1°50′W	Sudan-sahelian	rural	vegetables	28/08/09
Kombissiri	12°05′N; 1°35′W	Sudan-sahelian	rural	vegetables, cotton	28/08/09
Manga	11°66′N; 1°05′W	Sudan-sahelian	sub-urban	cereals, cotton	28/08/09
Koupela	12°20′N; 0°40′W	Sudan-sahelian	sub-urban	cotton	30/08/09
Fada	12°05′N; 3°55′E	Sudan-sahelian	sub-urban	cotton	30/08/09
Kompienga	11°30′N; 0°40E	Sudan-sahelian	rural	vegetables, cotton	30/09/09
Komiyenga	11°70′N; 0°60E	Sudan-sahelian	rural	cotton	30/09/09
Yamtenga	12°21′N ;1°31′W	Sudan-sahelian	peri-urban	swamp	28/08/09

Table 1. Main study sites across the country from where natural populations of *An. gambiae s.l.* were collected for susceptibility tests to insecticides in Burkina Faso.

Mosquitoes sampling: To evaluate the status of resistance of *An. gambiae s.l.* to insecticides in the three ecological zones of Burkina Faso, anopheline larvae were sampled in countrywide collections during the rainy season, from September to October. Larvae were collected at each locality from breeding sites such as gutters, tires, swallow wells and pools of standing water. Larvae were brought back to the insectary and reared to adulthood. When it was not possible to collect larvae because of the distance between the sampling site and the insectary or due to sampling constraints at the site such as excessive rainfall or flooding, alternative collections of adult mosquitoes were made using indoor aerosol insecticide spraying. *An. gambiae s.l.* were identified morphologically using standard identification keys of Gillies & Coetzee (1987). The results presented here summarized those of transversal studies in whole country 2000 and 2009 with particular focus on the period from September to October 2009.

Insecticide susceptibility test: Susceptibility test was performed on 2-3-day-old *An. gambiae s.l.* females provided by larva collections using the WHO standard vertical tube protocol. Three insecticide-impregnated papers were used: DDT 4%, permethrin 0.75% (cis:trans = 25:75), deltamethrin 0.05%, bendiocarb 0.1%, CM 0.04%, carbofuran 0.04% and fenithrotion 0.04%. Mosquitoes were tested against "Kisumu" a fully susceptible reference laboratory strain. Mortality controls were carried out by exposing both the "Kisumu" strain and wild populations from each site to non-insecticidal impregnated paper. After 1 h exposure,

mosquitoes were transferred into insecticide free tubes and maintained on sucrose solution. Final mortality was recorded 24 h after exposure. The threshold of susceptibility was fixed at 98% for the four active molecules according to the protocol of WHO (1998). Are considered as susceptible, suspected resistant and resistant populations with respectively 100 to 98%, 98 to 80 %and under 80% of mortality rates. Dead and survivor mosquitoes were grouped separately and stored on silicagel at -20°C for subsequent PCR analysis.

Molecular analysis: DNA extraction and PCR identification of the *An. gambiae* M and S and *An. arabienis*: Genomic DNA was extracted from individual mosquitoes according to a slightly modified version of the procedure described by Collins *et al*. (1987). After quantification of the extracted DNA, adults of *An. gambiae s.l.* tested in bioassay were processed by PCR for molecular identification of species of the *An. gambiae* complex and molecular forms respectively (Scott *et al*., 1993; Favia *et al*., 2001). Those survived or dead in bioassay were after processed in other PCR analysis for the detection of *kdr* and *ace-1*^{R} mutations. For *kdr* detection, a sub-sample of 30 mosquitoes per site of the permethrin/deltamethrin-tested specimens and those collected by indoor spraying were processed by PCR for prior species identification and molecular characterisation of M and S forms of *An. gambiae s.s.* according to Scott *et al.* (1993) and Favia *et al.* (2001) respectively. The frequency of the L1014F mutation in the same samples was determined by allele-specific PCR as described by Martinez-Torres *et al*. (1998).

A*ce-1*^{R} mutation was detected using the PCR-RFLP assay described by Weill *et al.* (2004) with minor modifications. Specific primers, *Ex3AGdir* (GATCGTGGACACCGTGTTCG) and *Ex3AGrev* (AGGATGGCCCGCTGGAACAG) were used in PCR reactions (25µl) containing 2.5µl of 10X *Taq* DNA polymerase buffer, 200µM of each desoxynucleoside triphosphate (dNTP), 0.1U of *Taq* DNA polymerase (Qiagen, France), 10pmol of each primer and approximately 1 to 10ng of DNA. PCR conditions included an initial denaturation step at 94°C for 5min followed by thirty five cycles of 94°C for 30s, 54°C for 30s and 72°C for 30s, with a final extension at 72°C for 5min. Fifteen microlitres of PCR product was digested with 5U of *Alu*I restriction enzyme (Promega, France) in a final volume of 25 µl at 37°C for 3 hours. Products were then analysed by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized under UV light.

Statistical analysis: The proportion of each species and molecular forms were compared between the study sites. The frequencies of *kdr* and *ace-1*^{R} mutations were calculated according to the formula p= 2AA+Aa/2n where AA was the number of homozygotes, Aa the number of heterozygotes and n the size of analyzed sample. It was compared between sites and between *An. gambiae* M and S molecular forms and *An. arabiensis* by chi square tests. The genotypic frequencies of ace-1R in mosquito populations were compared to Hardy-Weinberg expectations using the exact test procedures implemented in GenePOP (ver.3.4) software (Raymond & Rousset 1995)

3. Resistance to pyrethroids and organochlorine

Reports of resistance in mosquito vector populations in Burkina Faso appeared as early as the 1960 s, when *An. funestus* and *An. gambiae s.l.* populations that showed resistance to dieldrin and DDT, were described (Hamon *et al*., 1968a; Hamon *et al*., 1968b). More recent studies have confirmed that resistance to DDT4% is still prevailing with highest level in *An. gambiae s.l.* populations in Burkina Faso where also resistance to certain pyrethroids was increasingly reported (Diabaté *et al*., 2002, 2004a; Dabiré *et al*., 2009a). Indeed *An. gambiae s.l.*

populations were resistant to DDT4% in every part of the country and mortality rates below 60 % were observed at the country scale (Fig. 2). They were found also resistant to permethrin 0.75% in the Sudan climatic zone in the western region and also in several sites in the central part of Burkina Faso (Fig. 3). Surprisingly, except for the areas with a very long history of cotton cropping, the tested populations of *An. gambiae* remained susceptible to deltamethrin 0.05%, although decreased mortality values lead to suspect an emergence of resistance in the ongoing years (Dabiré *et al.*, 2009a). However, this result should be interpreted with caution as the resistance is a progressive process and recent data recorded in 2009-2010 showed 5 sites mostly located in the central region remained susceptible foci (Fig. 4).

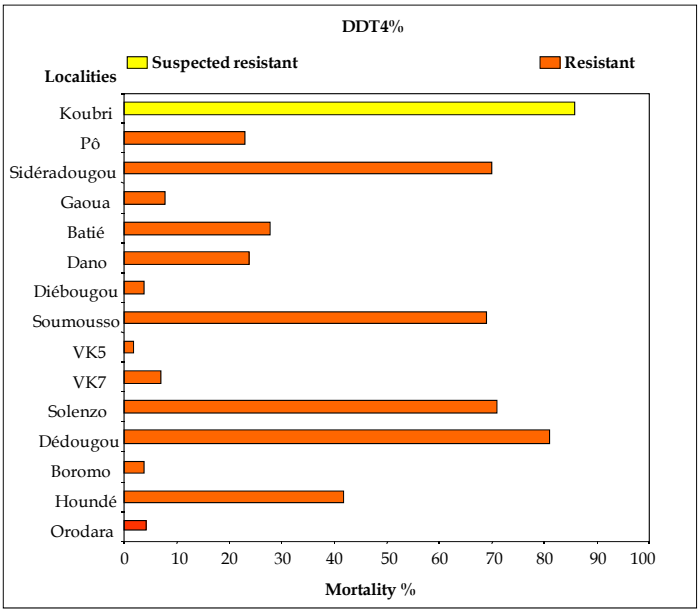

Fig. 2. Mortality rates of *Anopheles gambiae s.l.* populations to DDT 4% from Sudan, Sudan-sahelian and sahelian areas in Burkina Faso.

Resistance is the result of a limited number of physiological mechanisms; it is often monogenic and due to point mutations in a structural gene, gene amplification or changes in transcriptional regulation (Hollingworth & Dong, 2008). It results from the selection of mutant individuals able to survive and reproduce in presence of insecticide, the insecticide failing to disrupt the function of its target (Whalon *et al.*, 2008). The resistance phenotype to pyrethroids and DDT 4% observed in natural populations of *An. gambiae s.l.* was already attributed to a *kdr* mutation as it is the major mechanism involved in cross resistance to pyrethroids and DDT4% in West Africa (Chandre *et al.*, 1999; Diabaté *et al.*, 2002). Until recently, it was assumed that this mutation was the L1014F substitution in West Africa while the L1014S substitution was found in the East (Ranson *et al.*, 2000). However, we now know that both mutations coexist in some countries and are widely distributed throughout sub-Saharan continent and also in Benin and Burkina Faso (Verhaeghen *et al.*, 2006; Etang *et al.*, 2006; Djegbe *et al.*, 2011; Dabiré *et al.*, unpublished).

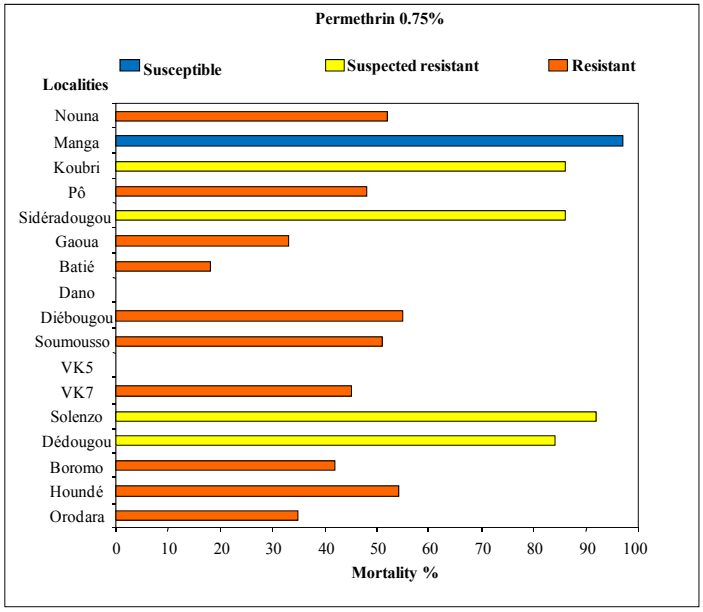

Fig. 3. Mortality rates of *An. gambiae s.l.* populations to permethrin 0.75 % from Sudan, Sudan-sahelian and sahelian areas in Burkina Faso.

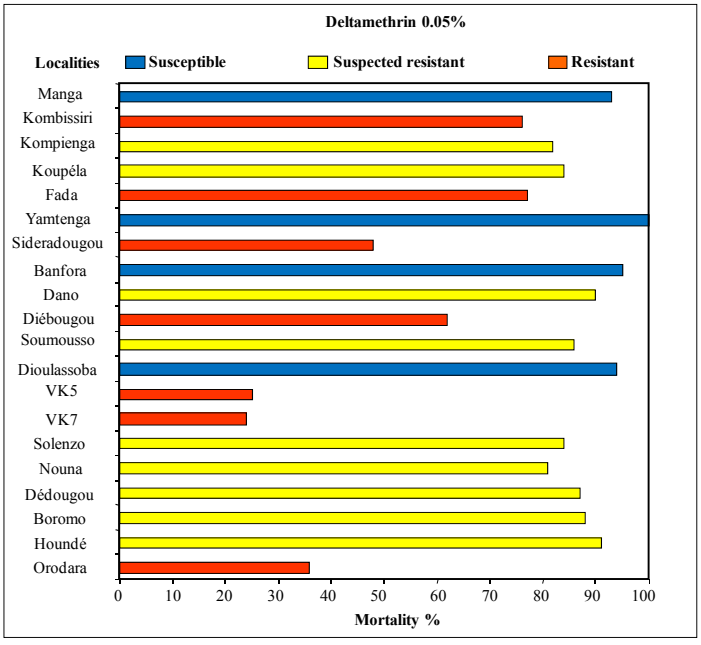

Fig. 4. Mortality rates of *An. gambiae s.l.* populations to deltamethrin 0.05 % from Sudan, Sudan-sahelian and sahelian areas in Burkina Faso.

In Burkina Faso, the frequency of the L1014F *kdr* mutation was first described in the S form of *An. gambiae s.s.* in high frequencies especially in the Western part of the country where the use of insecticides is intensive in agriculture (Chandre *et al*., 1999, Diabaté *et al*., 2002). But few years later it had been also found within the M form and was suspected to be the result of an introgression from the S form *An. gambiae s.s.* (Weill *et al*., 2000; Diabaté *et al*., 2003). Up to day the distribution of this mutation at the country scale is variable, ranging from 0.5 to 0.97 for the S form in the Sudanian region and decreasing in the Sudano-sahelian and Sahelian areas with averaged values fluctuating between 0.1 and 0.6. Compared to 2000 data (Diabaté *et al*., 2004a), the frequency of L1014F *kdr* mutation increased notably from 2004 to 2006 before getting stable around the fixation level in some localities (Fig. 5). As mentioned above, no *kdr* was detected in 1999 in the M form (Chandre *et al*., 1999). But early in 2000, the L1014F mutation was identified from few specimens of M form from rice growing area, peaking maximally at 0.04 (Diabaté *et al*., 2003). Nowadays the L1014F *kdr* has increased drastically in the M form with varying frequencies between climatic areas, and reaching high frequencies (0.93) in cotton growing belts with a geographic expansion to the sudano-sahelian region where it was formerly absent (Dabiré *et al*., 2009a). It has also increased in *An. arabiensis* (0.28) where it was formerly reported only from one specimen in 2002 (Diabaté *et al*., 2004b) (Fig. 6).

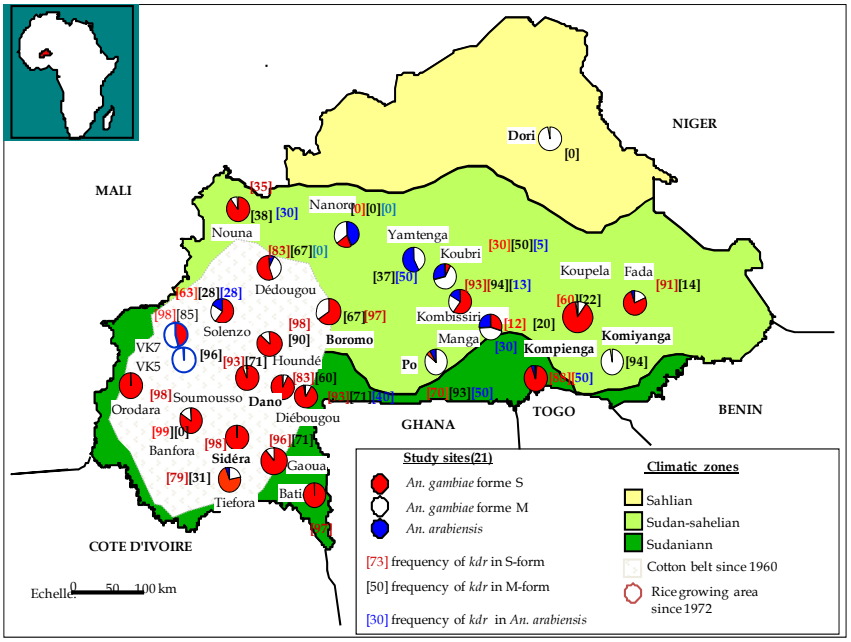

Fig. 5. Geographic distribution of L1014F *kdr* allele in *An. gambiae s.l.* populations inducing pyrethroids and DDT resistance profile in Burkina Faso in 2009 [numbers in bracket represent frequency of L1014F *kdr* allele frequencies].

Globally the distribution of DDT and pyrethroids resistance in regions of intensive cotton cultivation suggests that indirect selection pressures from the agricultural use of insecticides may be responsible for the development of resistance in *An. gambiae s.l.* populations. The

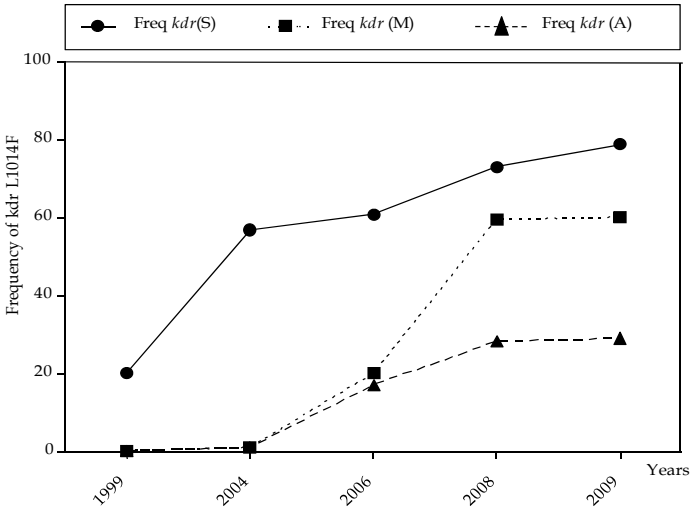

Fig. 6. Evolution of allelic frequencies (in percentages) of L1014F *kdr* in natural populations of *An. gambiae s.l.* from 1999 to 2009 in Burkina Faso[S: *An. gambiae* S form; M: *An. gambiae* M form, A: *An. arabiensis*].

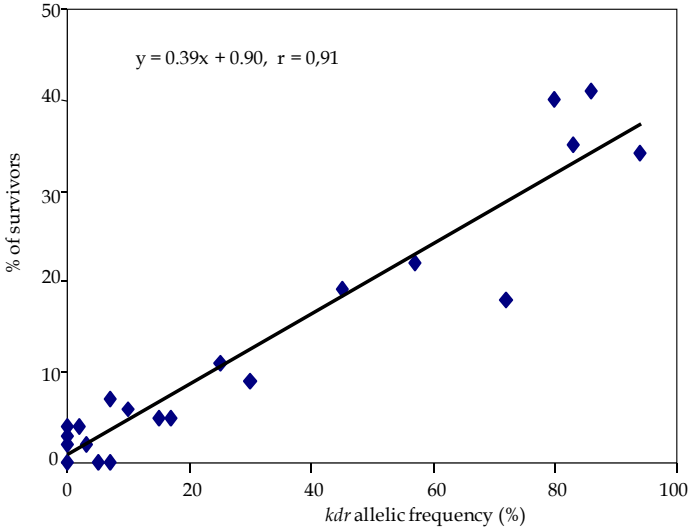

Fig. 7. Correlation between *kdr* frequency and mortality rates of *An. gambiae s.l.* tested with permethrin 0.75%.

mean frequency of the *kdr* allele was the highest in populations from the Sudanian zone where the lowest mortality rates to pyrethroids and DDT 4% were seen in bioassays. By contrast, the *kdr* allele frequency was lower in *An. gambiae s.s.* populations is central and eastern sites where cotton cultivation is recent. A majority of susceptible phenotypes were observed in wild populations of *An. gambiae* from these areas. In addition, the results of this

study suggest that the domestic use of insecticides may also exert a selection on *An. gambiae* populations that is secondary to that from the agricultural insecticides. Indeed all collections made in cities located outside the cotton belt showed high mortality rates and a relative low frequency of *kdr* compared to those of cotton belt. The correlation between the high frequencies of L1014F *kdr* mutation and the proportion of surviving individuals after DDT/pyrethroids exposures (figure 7) suggests that this mutation is the main mechanism of resistance to these insecticides.

4. Metabolic resistance

Most studies conducted in Burkina Faso have focused on the modification of target sites by mutation and did investigate the occurrence and the role of metabolic resistance in the observed resistance of *An. gambiae s.l.* Recent tests performed in Dioulassoba (an old central district of Bobo-Dioulasso crossed by the Houet river) showed that *An. arabiensis* from urban polluted breeding sites was resistant to DDT 4% but fully susceptible to pyrethroids and OP/CX, suggesting an existence of metabolic resistance probably GST which is more specific to DDT acting as the main resistance mechanism (Dabiré *et al*., unpubl.). Even more recently, preliminary results gathered only in VK7 (a sample from a rice growing area surrounded by cotton fields) showed an overexpression of detoxifying enzymes such as glutation-S-transferases, cytochrome P450 oxygenases in populations of *An. gambiae s.s.* with high *kdr* frequencies suggesting the existence of multi-resistance mechanisms to pyrethroids (Fig. 8A,B&C). But more investigations are needed to better address the role of metabolic components on the expression of resistance phenotypes observed in natural populations of *An. gambiae s.l.* especially in areas where insecticide pressure is high.

5. Resistance to organophosphates (OP) and carbamates (CX) and geographic distribution of *ace-1^R* mutations and duplicated *ace-1^D* allele

In Burkina Faso the resistance to OP/CX has been monitored since 2002 only in few sites of the Western areas of the country, and lately extended to the country scale since 2006. Although fenithrotion 0.4%, chlorpyriphos methyl (CM) 0.4%, carbosulfan 0.4% and bendiocarb 0.1 % were tested, the monitoring was well sustained only with bendiocarb 0.1% which was expected to be used in Burkina Faso indoor residual spraying to supplement the efficacy of ITNs especially in localities where *An. gambiae* is resistant to PY. Except for CM 0.4% for which *An. gambiae* populations were fully susceptible irrespectively of the locality, the other OP/CX mentioned above showed mortality rates ranging from 5% to 100% (Fig. 9). The lowest mortality rates were obtained with carbosulfan 0.4% (5%) and bendiocarb 0.1% (20%) especially in areas located in cotton belt such as Houndé, Orodara, Tiefora and Banfora. The susceptibility to bendiocarb 0.1% was also recorded in the central areas where cotton growing is recent (Fig. 10). From 2005 on, the detection of *ace-1^R* mutation involved in OP/CX resistance allowed to evaluate the distribution of this allele in field populations of *An. gambiae s.l.* The characterisation of this allele was based on a PCR-RFLP diagnostic (Weill *et al*., 2004) that allow the identification of the amino-acid substitution, from a glycine to a serine at the position 119, in the AChE1 catalytic site (G119S). In *Culex pipiens*, there is direct and indirect evidence that the resistance allele (*ace-1^R*) entails a large fitness cost, probably due to the mutated AChE1 having a much lower level of activity. Homozygous *ace-1^R* mosquitoes survive in the presence of insecticide, but are rapidly outcompeted in the

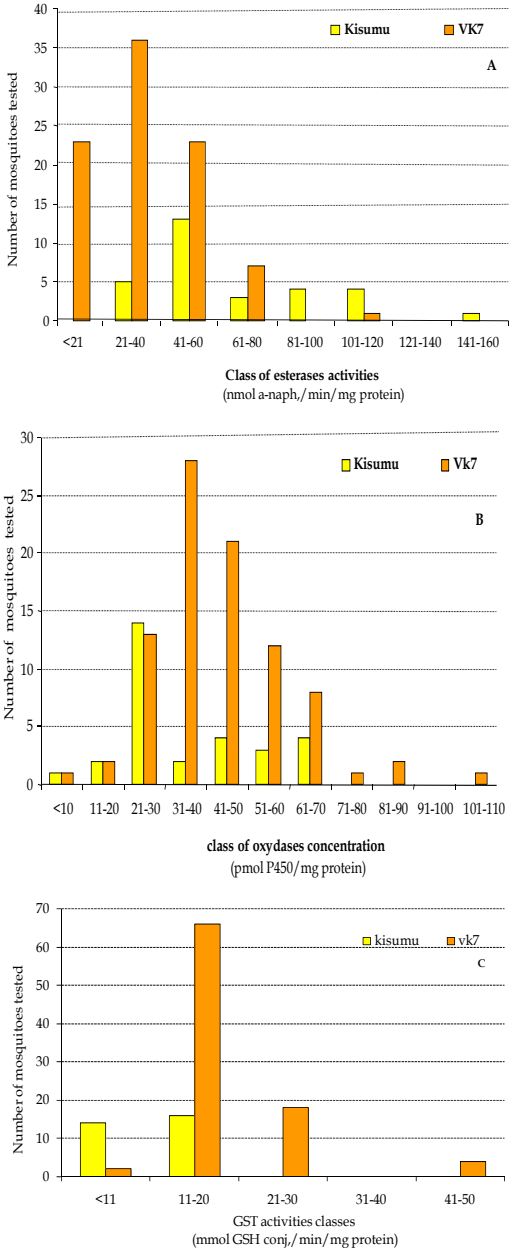

Fig. 8. Activity of detoxifying enzymes such as esterases (A), oxydases (B), and GST (C) in natural populations of *An. gambiae* from Vallée du Kou (VK7) compared to that of *An. gambiae* "Kisumu" (susceptible reference strain). Note the over-expression of oxydases and GST in the VK7 sample.

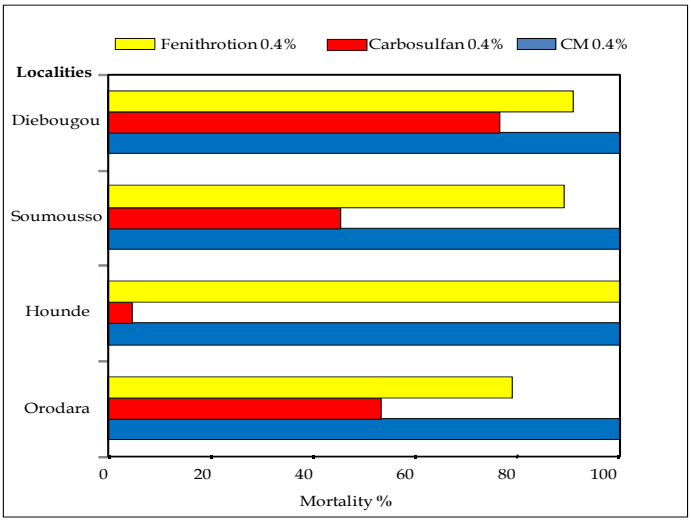

Fig. 9. Mortality rates of *An. gambiae s.l.* populations exposed to Chlorpyriphos methyl (CM) 0.04 %, carbofuran 0.04% and fenithrotion 0.04% from four sites located on the cotton belt in South west of Burkina Faso [100-98%=susceptible; 98-80%= suspected resistance; <80%=resistant].

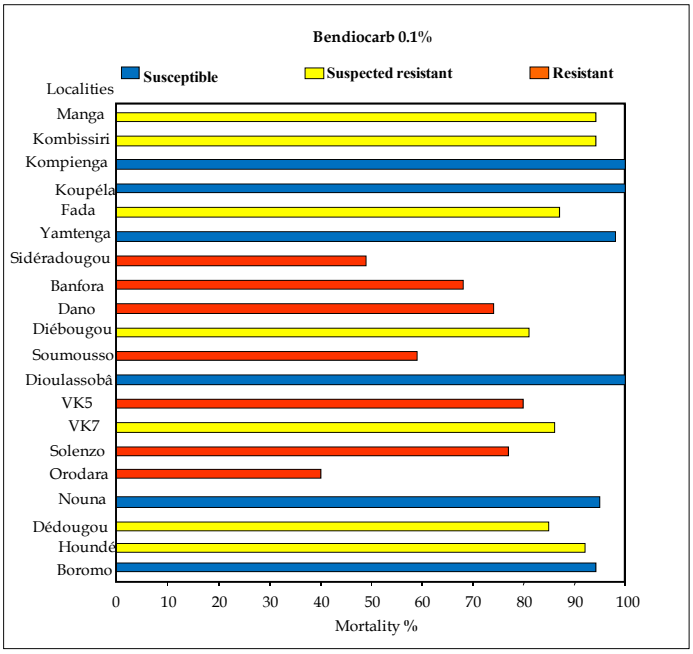

Fig. 10. Mortality rates of *An. gambiae s.l.* populations exposed to bendiocarb 0.1% from Sudan, Sudan-sahelian and sahelian areas in Burkina Faso.

absence of insecticide. There are evidences that the same phenomenon exists also in *An. gambiae s.s* (Djogbenou *et al*., 2010). Even though the results of bioassays were more recent (2009) we presented only the *ace-1*^{R} frequencies from 2006 to 2008 samples.

This mutation was distributed throughout the Sudan and Sudan-Sahelian localities reaching relative high frequencies (0.6) in the South-West, moderate frequencies (<50%) in the central region, and being absent in the Sahel. It was far more frequent in the S form than in the sympatric M mosquitoes (averaging in mean 0.32 for the S form *vs.* 0.036 for the M form) (Djogbenou *et al*., 2008a). Even though the *ace-1*^{R} mutation was spread across two climatic zones, it was recorded mostly in the cotton growing areas (Dabiré *et al*., 2009b). Although the *ace-1*^{R} mutation was less spread within the *An. gambiae* s.s. M form, the highest frequency (0.63) was recorded in this form at Houndé located just on the limit of the Sudan region in 2008 (Fig. 11). The observed genotypic frequencies were not significantly different from Hardy-Weinberg expectations at the 95% confidence level in populations from any site except in the *An. gambiae* s.s. S form population from Orodara, where an excess of heterozygotes was observed. S-form samples from a number of other sites also showed a higher than expected number of heterozygous genotypes including Banfora (expected 10, observed 13), Diebougou (expected 11, observed 16) and VK7 (expected 12, observed 17) although Hardy–Weinberg equilibrium was not rejected. These results suggest that a fitness cost is associated to this mutation (Labbé *et al*., 2007), but see the next paragraph. No *An. arabiensis* was detected up today carrying the *ace-1*^{R} mutation.

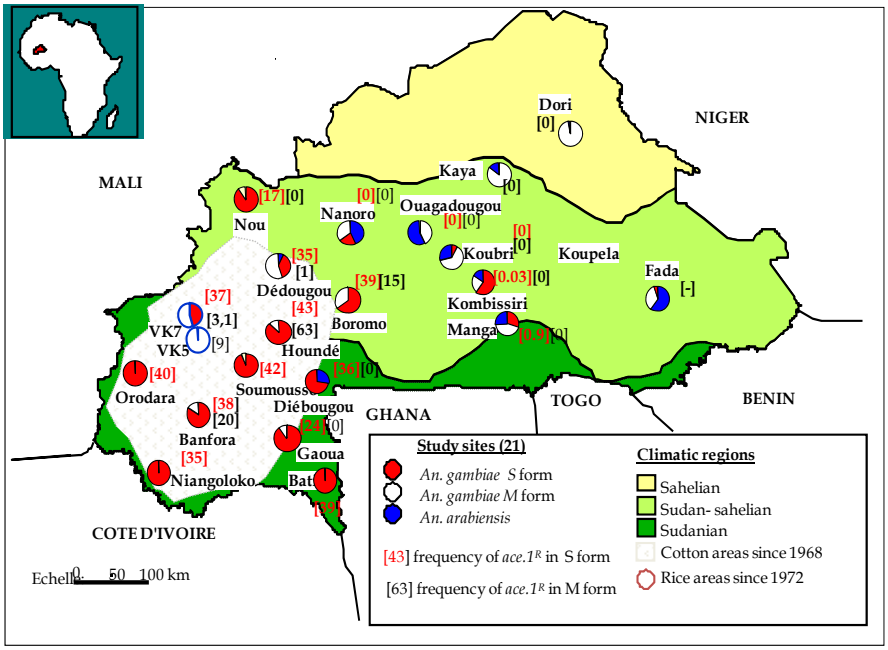

Fig. 11. Geographic distribution of *ace-1*^{R} allele in *An. gambiae s.l.* populations inducing OP/CX resistance profile in Burkina Faso in 2008 [numbers in bracket represent percentage of *ace-1*^{R} allele frequencies]. No *An. arabiensis* was found carrying ace-1R allele so we did not represent its frequency for this species.

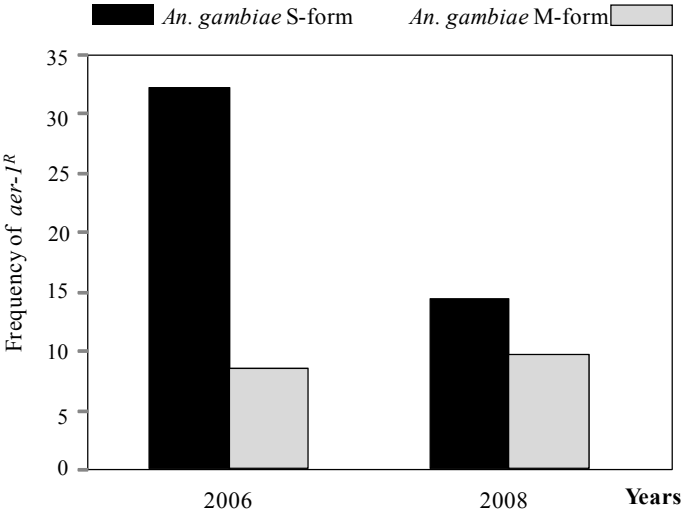

Fig. 12. Evolution of allelic frequencies of *ace-1*^{R} in natural populations of *An. gambiae s.s.* in Sudan area (cotton belt) from 2006 to 2008 in Burkina Faso.

The frequency evolution of this allele during the two years is not regular (fig.12), but considering only the Sudan area, it seems to decrease within the S form and increase slightly in the M form from 2006 to 2008 (Fig. 12). As no data existed before 2006, we did not know the trait of evolution of this allele in the past. That should greatly contribute to explain the inverse tendency of this allele within the two forms because the resistance pattern is complex in this area where excess of heterozygous for *ace-1*^{R} allele should probably co-exist with the duplication allele Ag-*ace-1*^{D} (see the next paragraph). However the allele frequencies in the two forms need to be compared statistically from a solid sample sizes. Regular monitoring of the same localities with the same protocols should give a better insight of the evolution of the G119S mutation of the *ace-1* gene. Data will have to be analyzed in relation with the possible coexistence of other resistance mechanisms such as *kdr* mutation or metabolic based resistance as well as with the existence of the duplicated allele Ag-*ace-1*^{D} which may decrease the fitness cost of this mutation (Berticat *et al*., 2008).

6. Duplication of *ace-1*^{D} allele in *An. gambiae s.s.* from Burkina Faso

The G119S mutation conferring resistance to organophosphates and carbamates was distributed throughout the Sudan and Sudan-sahelian correlated with the cotton growing areas. This mutation has been identified in the *ace-1*^{R} allele, and recently in a "duplicated allele" (*ace-1*^{D}), putting in tandem a susceptible and a resistant copy of *ace-1* on the same chromosome. The ace-*1*^{D} has been recorded in field populations of *An. gambiae* M and S forms and was shown to have come from the same duplication event in both forms (Djogbenou *et al*., 2008b). A unique *ace-1*^{D} allele has been observed in Côte d'Ivoire and Burkina Faso, with an estimated frequency >50% in some populations (Djogbenou *et al*., 2009).

In Burkina Faso, the *ace-1*^{D} allele frequency could reach 50% and is mainly present in the S form principally in the old cotton belt in the South West. The duplicated allele was also

observed on the littoral of Ivory Coast with high frequencies in the M form, and may be present at a low frequency in Benin and Togo (Djogbenou *et al*., 2010) (Fig. 13).
If, as suspected by Labbé *et al.* (2007), *ace-1*^{D} allele has a lower fitness cost than *ace-1*^{R}, it would increase dramatically the diffusion of OP and CX resistance in *An. gambiae* s.s. natural populations.
Presently, there is no simple test to characterized the duplicated allele *ace-1*^{D} as diagnostic tests do not discriminate between heterozygotes *ace-1*^{S}/*ace-1*^{R} and genotypes with the *ace-1*^{D} allele. Formal identification of *ace-1*^{D} in a field female thus necessitate to cross this female with a susceptible male, and screen its offspring for CX resistance (see Labbé *et al*. 2007 for detailed procedure). This is clearly not possible on a large number of specimens and to better address the role and the impact of the *ace-1* duplication in resistance schemes, it appears urgent to build an *ace-1*^{D} homozygous laboratory strain to investigate how the duplication modifies the fitness of its carriers.

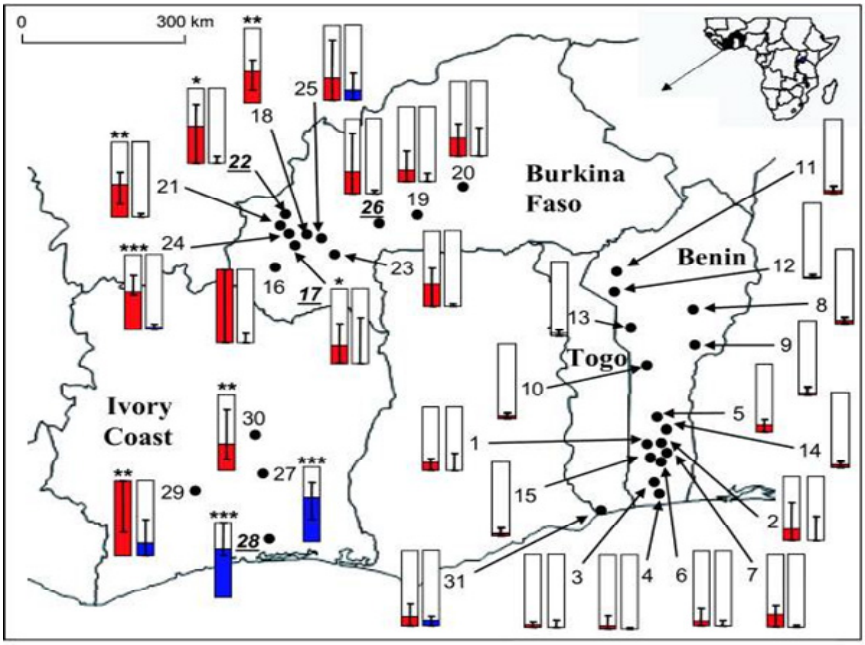

Fig. 13. Ag- *ace-1*^{D} frequency in Western Africa. The frequency is given for each *An. gambiae* molecular form: S (red) and M blue). Samples in which *ace-1*^{D} was detected by molecular analysis are bolded and underlined. Significant presence of the duplicated allele (before Bonferroni correction) is given with * for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$.
(Figure from Djogbenou *et al*., 2009 in Malaria Journal)

7. Multiresistance status in natural populations of *An. gambiae s.l.*: the coexistence of *kdr* and *ace-1^R* mutations

It was in 2005 when the detection of *ace-1^R* (G119S) mutation was systematically performed for the first time in *An. gambiae s.s.* natural populations from Burkina Faso (Dabiré *et al*., 2008). This mutation was found together with *kdr* mutation within the same populations. That suggests the existence of multiresistance mechanisms occurring in the same populations. The same individuals were found carrying the two genes but the *kdr* always appeared as homozygous (*kdr*^{R}/*kdr*^{R}). The functional links of the two genes needed to be further investigated (Dabire *et al*., 2008). Indeed as indicated by the results of bioassays, the occurrence of such multiresistance mechanisms should explain why *An. gambiae s.s.* populations are becoming resistant to all classes of insecticides especially in the South west parts of the country. The individuals carrying the two genes appeared to be phenotypically more resistant to pyrethroids and bendiocarb than those carrying only *kdr* or *ace-1^R*, respectively. Such a synergy between *kdr* and *ace-1^R* has been observed in *Culex pipiens* (Berticat *et al*., 2008). Assuming that the *ace-1^R* is associated to a high fitness cost (Djogbenou *et al*., 2010), it should be interesting to investigate how the *kdr* mutation influences the fitness cost related to *ace-1^R*. Also, it was previously shown in *Culex pipiens* that mosquitoes carrying both *kdr* and *ace-1^R* mutations suffer less cost than the one carrying only *ace-1^R* (Berticat *et al*., 2008). Both this synergy between pyrethroids and OP/CX resistance mechanisms and the spread of the *ace-1^D* allele in natural populations of *An. gambiae s.s.* could largely hamper the expected results of using OP/CX as alternative insecticides to the PYR becoming ineffective by the presence of *kdr*. It is crucial to build laboratory colonies carrying the two mutations from which the benefit of changing insecticides could be properly tested. More recently, in 2011, we also recorded metabolic based resistance in *An. gambiae* from Burkina Faso. Even though these results are preliminary, they further complicate the pattern of resistance in this country, and may represent a dramatic threat for malaria vector control in the near future.

In conclusion, all these aspects need to be properly addressed by fine fundamental research to decrypt the link between the resistance schemes and to give sense in vector control point of view.

8. Other malaria vectors

Anopheles funestus belongs to a group of no less than nine species that are difficult to distinguish based solely on morphological characters of a single life stage (Gillies and Coetzee 1987, Harbach 1994). Species identification difficulties have been recently addressed by molecular techniques based on the polymerase chain reaction (PCR) by using a cocktail of species-specific primers permitting the identification of the six most common species of the group (Koekemoer *et al*., 2002). Recent analyses of rDNA sequences (Cohuet *et al*., 2003) revealed the occurrence, in West and Central Africa, of a new taxon morphologically related to *An. rivulorum* Leeson, which is provisionally named *An. rivulorum*-like, thereby enlarging the number of members of the *An. funestus* group to 10 species. Among all the members of the *funestus* group, *An. funestus* s.s. is the most anthropophilic species, and it is considered as the only major malaria vector (Coetzee & Fontenille, 2004), although in a Tanzanian village the circumsporozoite protein of *Plasmodium falciparum* was detected by immunological techniques in some *An. rivulorum* specimens (Wilkes *et al*. 1996).

An. funestus like other malaria vectors is controlled by the use of insecticides such as insecticide treated materials or as indoor residual spraying (IRS). Unfortunately, *An. funestus* is increasingly developing resistance across Africa to different classes of insecticides used in public health, such as PYR, CX and DDT (Brooke *et al*., 2001; Casimiro *et al*., 2006; Cuamba *et al*., 2010; Morgan *et al*., 2010). There are alternative agrochemicals, such as fipronil that could be introduced but the potential for cross-resistance from existing mechanisms segregating in field populations needs to be more investigated.

The insecticide resistance in *An. funestus* populations was early recorded from Burkina Faso, where resistance was found to dieldrin, a cyclodiene abundantly used in Africa in the 1960s for cotton crop protection but also for malaria vector control (Hamon *et al*., 1968b). Dieldrin resistance was also reported in *An. funestus* from Cameroon, Benin, Nigeria and Mali (Service, 1960; Toure, 1982; Brown, 1986). Recent studies have shown that *An. funestus* remains fully susceptible to all tested insecticides (DDT, PYR, OP/CX) except to dieldrin for which resistance remains high despite the fact that cyclodienes are no longer used in public health control programs (Dabire *et al*., 2007). But the distribution of this resistance across the rest of the continent is unknown and need to be clarified. The understanding of factors explaining the persistence of high levels of resistance against cyclodienes in *An. funestus* as well as the geographical distribution of this resistance across the continent has been recently addressed by Wondji *et al*., 2011. These studies indicated that Rdl^R mutation extensively reported in West and Central Africa should sustain dieldrin resistance in such *An. funestus* populations.

9. Pesticide pressures on disease vectors are from multiple origins

The question that remains to be clearly identified is the origin of insecticide pressures that select the resistances observed in mosquitoes from sub-Saharan Africa. In Burkina Faso, the emergence of the $ace\text{-}1^R$ mutation in *An. gambiae s.s.* populations is also associated with the insecticide treatment history with OP and CX of cotton. Since the mid 1990s and until recently a pest management strategy including four windows of treatment per cropping cycle using pyrethroids, OP/CX (such as chlorpyrifos, profenofos and trizophos) and organochlorines had been adopted in order to manage the pyrethroid-resistance of *Helicoverpa armigera* and *Bemisia tabaci* that emerged throughout the cotton belt. Some bioassays performed in 2003 on *An. gambiae* populations from four sites located in the cotton belt of western Burkina Faso revealed early resistance against CX and OP insecticides pre-empting the discovery of the genetic resistance mechanism revealed in further studies. In a previous study the agricultural use of insecticides was already implicated in the development of resistance to pyrethroids in *An. gambiae s.l.* populations. Then the geographical distribution of resistance decreased in *An. gambiae s.l.* populations from the Sudan savannah to Sahelian areas and the highest levels of resistance were found in sites of cotton cultivation. The areas under cotton cultivation have expanded dramatically in the last ten years (210,000 ha in 1996 to more than 520,000 ha in 2005). A corresponding increase in the level of insecticide use has also been reported reaching more than $3x10^6$ litres of pesticide per cropping campaign. Furthermore, a clear knowledge concerning the practices of populations regarding the uses of insecticides in Africa is required.

10. Insecticide resistance and malaria vector control

Although several studies in Ivory Coast and Burkina Faso had shown that ITNs may still achieve good control of PYR-target resistant *An. gambiae s.s.* populations (Darriet *et al*., 2000; Henry *et al*., 2005; Dabiré *et al*., 2006), recent results from experimental hut trials conducted in Southern Benin with lambdacyhalothrin (PYR) suggested that such ITNs may fail to control these field populations (Ngessan *et al*., 2007). Failure of indoor residual house spraying (IRS) with deltamethrin (PYR) had also occurred in South Africa where the malaria vector *An. funestus* had developed PYR-metabolic resistance. A recent study in Bioko Island (Equatorial Guinea) reported a failure of indoor residual spraying with deltamethrin on *An. gambiae* populations of the M molecular form carrying the Leu-Phe *kdr* mutation at a high frequency. These *kdr*-pyrethroid resistant populations were controlled after the introduction of a carbamate insecticide in IRS (Sharp *et al*., 2007). Thus the malaria outbreak in this country was only brought under control after reversion to DDT spraying (organochlorine insecticide).

A concern for the potential use of OP and CX as alternative for PYR is that target and metabolic resistances to these insecticides are already present in some *An. gambiae s.s.* populations in West Africa especially in Burkina Faso. Several studies have suggested that the use of agricultural pesticides, especially for cotton but also for vegetable crops, favored the emergence and facilitated the spread of insecticide resistance within mosquito populations. Other studies have given evidence for the selection of *kdr* alleles associated with the use of pyrethroids in ITNs and other domestic strategies of personal protection, especially in Kenya and Niger (Czeher *et al*., 2008). In countries supported by PMI (President Initiative against Malaria) such as Ghana, Senegal, Mali, Benin and Liberia in West Africa the large-scale pilot interventions implemented with bendiocarb 400mg/m^2 in IRS could also contribute to select the OP/CX resistance. However, as no global health control program in Africa used OP and CX for mosquito control, it is necessary to clearly identify the origin of insecticide pressure that select these resistances.

11. Conclusions

Reports of insecticide resistance in malaria vectors in West Africa especially in Burkina Faso indicate that insecticide resistance increases year after year and highlight the threat to the effectiveness of vector control strategies. In fact *An. gambiae s.l.* populations in Burkina Faso, and more broadly in West Africa, have evolved resistance to many of the insecticides classes used for vector control. Resistance may be conferred by target-site insensitivity such as *kdr* and *ace-1^R*, other metabolic mechanisms or a combination of all as *kdr* and *ace-1^R* resistance mechanisms occur concomitantly in the same populations of *An. gambiae* s.s. in the South-Western region of the country.

In conclusion the geographical distribution of insecticide resistance in *An. gambiae s.l.* populations was found in sites of cotton cultivation and vegetable in urban settlement that has expanded dramatically in the last ten years. But the role of agriculture in the selection of resistance in natural mosquito's populations needs to be clarified, both in terms of insecticides usage and quantity in order to devise strategies that may help to reduce the extension of resistance. Until the discovery of new insecticides or using new formulations of existing insecticides and also the use of genetically modified mosquitoes (GMM) and sterilised males techniques (SIT), it is crucial to integrate the regional vector resistance status

in the implementation of control interventions that will preserve a long term efficacy of these vector control tools.

Unfortunately reports of insecticide resistance in vector populations increase year by year and could jeopardize malaria vector control based on the use of insecticides. The use of insecticides for bednets impregnation or for IRS represents the primary means for malaria prevention worldwide. However the efficacy of such tools has been evaluated in areas where vectors are susceptible to insecticides. Moreover, mosquito resistance is not only due to the insecticides used for mosquito control, but to the many pesticide pollutions present in their environment which are generated by a large variety of human activities necessitating insect control for agriculture (large cultures, fruits and vegetables), animal and other household protections. These pollutions may dramatically affect resistance genes dynamics and threaten these strategies.

The overall pesticide pressures that select resistance in mosquitoes need to be clarified, both in terms of insecticides usage and quantity. It is also crucial to improve our knowledge on the practices of people regarding the use of insecticides and the reasons underlying their decision process based on social and cultural contexts.

Malaria vector control programs require up-to-date information on the distribution and composition of mosquito vector populations and the susceptibility of these populations to the insecticides used for control.

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13. References

[1] Awolola TS, Oyewole IO, Amajoh CN, Idowu ET, Ajayi MB, Oduala A, *et al*., 2005. Distribution of the molecular forms of *Anopheles gambiae* and pyrethroids knock down resistance gene in Nigeria. *Acta Tropica* 95: 204-09

[2] Beier J, Keating J, Githure J, Macdonald M, Impoinvil D, Novak R, 2008. Integrated vector management for malaria control. *Malaria Journal* 7: S4.

[3] Berticat C, Bonnet J, Duchon S, Agnew P, Weill M, Corbel V, 2008. Costs and benefits of multiple resistance to insecticides for *Culex quinquefasciatus* mosquitoes. *BMC Evolutionary Biology* 8.

[4] Brogdon WG, McAllister JC, 1998. Insecticide resistance and vector control. *Emerging infectious Diseases* 4: 605-613.

[5] Brooke BD, Kloke G, Hunt RH, Koekemoer LL, Temu EA, Taylor ME, Small G, Hemingway J, Coetzee M, 2001. Bioassay and biochemical analyses of insecticide resistance in southern African *Anopheles funestus* (Diptera: Culicidae). *Bull Entomol Res* 91: 265-272.

[6] Brown AWA, 1986. Insecticide resistance in mosquitoes: a pragmatic review. J *Am Mosq Control Assoc* 2: 123-140.

[7] Casimiro S, Coleman M, Mohloai P, Hemingway J, Sharp B, 2006. Insecticide resistance in *Anopheles funestus* (Diptera: Culicidae) from Mozambique. *J Med Entomol* 43: 267-275.

[8] Chandre F, Manguin S, Brengues C, Dossou-Yovo J, Darriet F, Diabaté A, Faye O, Mouchet J, Guillet P, 1999. Status of pyrethroid resistance in *Anopheles gambiae* sensu lato. *Bull World Health Organ* 77: 230-4.

[9] Coetzee M, Fontenille D, 2004. Advances in the study of *Anopheles funestus*, a major vector of malaria in Africa. *Insect Biochem Mol Biol* 34: 599-605.

[10] Collins FH, Mendez MA, Rasmussen MO, Mehaffey PC, Besansky NJ, Finnerty V, 1987. A ribosomal RNA gene probe differentiates member species of the *Anopheles gambiae* complex. *Am J Trop Med Hyg* 37: 37-41.

[11] Cohuet A, Simard F, Toto JC, Kengne P, Coetzee M, Fontenille D, 2003. Species identification within the *Anopheles funestus* group of malaria vectors in Cameroon and evidence for a new species. *Am J Trop Hyg* 69: 200-205.

[12] Cuamba N, Morgan JC, Irving H, Steven A, Wondji CS, 2010. High level of pyrethroid resistance in an *Anopheles funestus* population of the Chokwe District in Mozambique. *PLoS One* 5, e11010.

[13] Czeher C., Labbo R., Arzika I., Duchemin J-B., 2008. Evidence of increasing Leu-Phe knockdown resistance mutation in *Anopheles gambiae* from Niger following a nationwide long-lasting insecticide-treated nets implementation. *Malaria Journal*

[14] Dabiré KR, Diabaté A, Namountougou M, Toé KH, Ouari A, Kengne P, Bass C, Baldet T, 2009a: Distribution of pyrethroid and DDT resistance and the L1014F *kdr* mutation in *Anopheles gambiae s.l.* from Burkina Faso (West Africa). *Tran R Soc Trop Med Hyg* 103: 1113-1120

[15] Dabiré K.R, Diabaté A., Namountougou M., Djogbenou L., Kengne P., Ouédraogo J-B., Simard F., Bass C., Baldet T., 2009b. The distribution of insensitive acetylcholinesterase (*ace*-1^R) in *Anopheles gambiae* s.l. populations from Burkina Faso (West Africa). *Tropical Medicine and International Health* 14 (4): 396-403

[16] Dabiré KR, Diabaté A, Baldet T, Paré L, Guiguemdé TR, Ouédraogo JB, Skovmand O, 2006. Personal protection of long lasting insecticide-treated nets in areas of *Anopheles gambiae* ss resistance to pyrethroids. *Malaria Journal* 5-12 doi: 10.1186/1475-2875-5-12.

[17] Dabiré KR, Baldet T, Diabaté A, Dia I, Costantini C, Cohuet A, Guiguemde TR, Fontenille D, 2007. *Anopheles funestus* (Diptera: Culicidae) in a humid savannah area of western Burkina Faso: bionomics, insecticide resistance status, and role in malaria transmission. *J Med Entomol* 44: 990-997.

[18] Dabiré KR, Diabaté A, Djogbenou L, Ouari A, N'Guessan R, Ouédraogo JB, Hougard JM, Chandre F, Baldet T, 2008. Dynamics of multiple insecticide resistance in the malaria vector *Anopheles gambiae* in a rice growing area in South-Western Burkina Faso. *Malaria Journal* 7: 188.

[19] Darriet F, N'Guessan R, Koffi AA, Konan L, Doannio JM, Chandre F, Carnevale P, 2000. Impact of pyrethrin resistance on the efficacy of impregnated mosquito nets in the prevention of malaria: results of tests in experimental cases with deltamethrin SC. *Bull Soc Pathol Exot* 93: 131-4

[20] Della torre A, Tu Z, Petrarca V, 2005. On the distribution and genetic differentiation of *Anopheles gambiae* ss molecular forms. *Insect Bioch Mol Biol*, 35:7055-69

[21] Diabaté A, Brengues C, Baldet T, Dabiré KR, Hougard JM, Akogbeto M, Kengne P, Simard F, Guillet P, Chandre F, 2004a. The spread of the Leu-Phe *kdr* mutation through *Anopheles gambiae* complex in Burkina Faso: genetic introgression and de *novo* phenomena. *Trop Med Int Health* 9: 1267-73

[22] Diabaté A, Baldet T, Chandre F, Akogbeto M, Darriet F, Brengues C, Guillet P, Hemingway J, Small GJ, Hougard JM, 2002. The role of agricultural use of insecticides in resistance to pyrethroids in *Anopheles gambiae* sl in Burkina Faso. *Am J Trop Med Hyg* 67: 617-22

[23] Diabaté A., Baldet T., Chandre F., Dabiré K.R., Kengne P., Guiguemdé T.R., Simard F., Guillet P., Heminway J., Hougard J.M., 2003. KDR mutation, a genetic marker to assess events of introgression between the molecular M and S forms of *Anopheles gambiae* (Diptera: Culicidae) in the tropical savannah area of West Africa. *J Med Entomol*, 40:195-198

[24] Diabaté A, Baldet T, Chandre F, Dabiré KR, Simard F, Ouédraogo JB, Guillet P, Hougard JM, 2004b. First report of kdr mutation in *Anopheles arabiensis* from Burkina Faso, West Africa. *J Am Mosq Control Ass* 20: 195-196.

[25] Djegbe I., Boussari O., Sidick A., Martin T., Ranson H., Chandre F., Akogbeto M., Corbel V., 2011. Dynamics of insecticide resistance in malaria vectors in Benin: first evidence of the presence of L1014S *kdr* mutation in *Anopheles gambiae* from West Africa. *Malaria Journal*, 10:261 http://www.malariajournal.com/content/10/1/261

[26] Djogbenou L, Labbe P, Chandre F, Pasteur N, Weill M, 2009. Ace-1 duplication in *Anopheles gambiae*: a challenge for malaria control. *Malaria Journal* 8:70.

[27] Djogbenou L, Chandre F, Berthomieu A, Dabire KR, Koffi A, Alout H, Weill M, 2008. Evidence of introgression of the *ace-1*^{R} mutation and of the *ace-1* duplication in West African *Anopheles gambiae* s. s. *PLoS ONE,* 3, e2172.

[28] Djogbenou L, Noel V, Agnew P, 2010. Costs of insensitive acetylcholinesterase insecticide resistance for the malaria vector *Anopheles gambiae* homozygous for the G119S mutation. *Malaria Journal*, 9:12.

[29] Etang J, Fonjo E, Chandre F, Morlais I, Brengues C, Nwane P, Chouaibou M, Ndjemai H, Simard F, 2006. Short report: First report of knockdown mutations in the malaria vector *Anopheles gambiae* from Cameroon. *Am J Trop Med Hyg* 74: 795-797.

[30] Fanello C, Akogbeto M, della Torre A, 2000. Distribution of the knockdown resistance gene (*kdr*) in *Anopheles gambiae* s.l. from Benin. *Trans R Soc Trop Med Hy* 94: 132.

[31] Favia G, Lanfrancotti A, Spanos L, Sideén-Kiamos I, Louis C, 2001. Molecular characterization of ribosomal DNA polymorphisms discriminating among chromosomal forms of *Anopheles gambiae* ss. *Insect Mol Biol* 10: 5-3

[32] Gillies MT, Coetzee M, 1987. A supplement to the Anophelinae of Africa south of the Sahara (Afrotropical region). Publications of the South African Institute for Medical Research, 1987, no. 55. SAIMR, Johannesburg.

[33] Hamon J, Subra R, Venard P, Coz J, Brengues J, 1968a. Présence dans le Sud-Ouest de la Haute Volta de populations d'*Anopheles funestus* Giles résistantes à la dieldrine. *Med Trop* 28: 221-26

[34] Hamon J, Subra R, Sales S, Coz J, 1968b. Présence dans le Sud-Ouest de la Haute Volta de populations d'*Anopheles gambiae* "A" résistantes au DDT. *Med Trop* 28: 524-28

[35] Kaminski J, 2007. Reforme de la filière cotonnière burkinabé- Retour sur dix ans de mutations: Analyse des impacts économiques et sociaux sur les producteurs et implications des organisations agricoles. Rapport Université de Toulouse 2007, 96p

[36] Harbach RE, 1994. Review of internal classiÞcation of the genus *Anopheles* (Diptera: Culicidae): the foundation for comparative systematic and phylogenetic research. *Bull Entomol Res* 84: 331-342.

[37] Henry MC, Assi SB, Rogier C, Dossou-Yovo J, Chandre F, Guillet P, Carnevale P, 2005. Protective Efficacy of Lambda-Cyhalothrin Treated Nets in *Anopheles gambiae* Pyrethroid Resistance Areas of Cote d'Ivoire. *Am J Trop Med Hyg* 73: 859-864.

[38] Koekemoer LL, Weeto MM, Kamau L, Hunt RH, Coetzee M, 2002. A cocktail polymerase chain reaction (PCR) assay to identify members of the *Anopheles funestus* (Diptera: Culicidae) group. *Am J Trop Med Hyg* 66: 804-811.

[39] Hollingworth RM, Dong K, 2008. The biochemical and molecular genetic basis of resistance in arthropods. Pp. 192 *in* M. E. Whalon, D. Mota-Sanchez, and R. M. Hollingworth, eds. Global pesticide resistance in arthropods. CAB International, Cambridge, MA.

[40] Labbé P, Berthomieu A, Berticat C, Alout H, Raymond M, Lenormand T, Weill M, 2007. Independent duplications of the acetylcholinesterase gene conferring insecticide resistance in the mosquito *Culex pipiens*. *Molecular Biology and Evolution* 24: 1056–1067.

[41] Lemasson JJ, Fontenille D, Lochouarn L, Dia I, Simard F, Ba K, Diop A, Diatta M, Molez JF, 1997. Comparison of behavior and vector efficency of *Anopheles gambiae* and *An. arabiensis* (Dipetra: Culidae) in Barkedji, a Sahelian area of Senegal. *J Med Entomol* 34: 396-403

[42] Martinez-Torres D, Chandre F, Williamson MS, Darriet F, Berge JB, Devonshire AL, Guillet P, Pasteur N, Pauron D, 1998. Molecular characterization of pyrethroid knockdown resistance (*kdr*) in the major malaria vector *Anopheles gambiae* ss. *Insect Mol Biol* 7: 179-84

[43] Morgan JC, Irving H, Okedi LM, Steven A, Wondji CS, 2010. Pyrethroid resistance in an *Anopheles funestus* population from Uganda. *PLoS One 5*, e11872.

[44] Muller P, Chouaibou M, Pignatelli P, Etang J, Walker ED, Donnelly MJ, Simard F, Ranson H, 2008. Pyrethroid tolerance is associated with elevated expression of antioxidants and agricultural practice in *Anopheles arabiensis* sampled from an area of cotton fields in Northern Cameroon. *Molecular Ecology* 17:1145-1155.

[45] Nauen R, 2007. Insecticide resistance in disease vectors of public health importance. *Pest Management Science* 63: 628-633.

[46] Nguessan R, Corbel V, Akogbeto M, Rowland M, 2007. Reduced Efficacy of Insecticide treated Nets and Indoor Residual Spraying for Malaria Control in Pyrethroid Resistance Area, Benin. *Emerging Infectious Diseases*, 2 www.cdc.gov/eid

[47] Ranson H, Jensen B, Vulule JM, Wang X, Hemingway J, Collins FH, 2000. Identification of a point mutation in the voltage-gated sodium channel gene of Kenya *Anopheles*

gambiae associated with resistance to DDT and pyrethroids. *Insect Mol Biol* 95: 491-97

[48] Raymond M, Rousset F, 1996. GENEPOP (version 1.2): Population genetics software for exact tests and ecumenicism. *J Heredity* 86: 248-49.

[49] Rogan W. J., Chen A., 2005. Health risks and benefits of bis (4-chlorophenyl)-1,1,1-trichloroethane (DDT). *The Lancet* 366:763-773.

[50] Sharp B.L., Ridl F.C., Govenderi D., Kuklinski J., Kleinschmidt I., 2007. Malaria vector control by indoor residual insecticide spraying on the tropical island of Bioko, Equatorial Guinea. *Malaria Journal*, 6:52 doi:10.1186/1475-2875-6-52

[51] Scott JA, Brogdon WG, Collins FH, 1993. Identification of single specimens of *An. gambiae* complex by polymerase chain reaction. *Am J Trop Med Hyg* 49: 520-29

[52] Service MW, 1960. A taxonomic study of *Anopheles funestus* Giles (Diptera: Culicidae) from southern and northern Nigeria, with notes on its varieties and synonyms. *Proc Entomo Soc Lond Ser B* 29: 77-84.

[53] Touré YT, 1982. Study of *Anopheles funestus* and *Anopheles gambiae* s.l. susceptibility to insecticides in a rural area of Sudan savanna in Mali. *Cahiers ORSTOM, Ser Entomologie Medicale Parasitologie* 20: 125-131.

[54] Touré YT, Petrarca V, Traoré SF, Coulibaly A, Maiga HM, Sankaré O, Sow M, Di Decco MA, Coluzzi M, 1998. The distribution and inversion polymorphism of chromosomally recognised taxa of the *Anopheles gambiae* complex in Mali, West Africa. *Parassitologia* 40: 477-511

[55] Verhaeghen K, Van Bortel W, Roelants P, Backeljau T, Coosemans M, 2006. Detection of the East and West African *kdr* mutation in *Anopheles gambiae* and *Anopheles arabiensis* from Uganda using a new assay based on FRET/Melt Curve analysis. *Malaria Journal* 5:16

[56] Weill M, Chandre F, Brengues C, Manguin C, Akogbeto M, Pasteur N, Guillet P, Raymond M, 2000. The *kdr* mutation occurs in the Mopti form of *Anopheles gambiae* s.s. through introgression. Insect Mol. Biol. 9:451-455.

[57] Weill M, Lutfalla G, Mogensen K, Chandre F, Berthomieu A, Berticat C, Pasteur N, Philips A, Fort P, Raymond M, 2003. Insecticide resistance in mosquito vectors. *Nature* 423: 136-137.

[58] Weill M, Malcolm C, Chandre F, Mogensen K, Berthomieu A, Marquine M, Raymond M, 2004. The unique mutation in *ace-1* giving high insecticide resistance is easily detectable in mosquito vectors. *Insect Molecular Biology* 13:1-7.

[59] Whalon ME, Mota-Sanchez D, Hollingworth RM, 2008. Analysis of global pesticide resistance in arthropods. Pp. 192 *in* M. E. Whalon D, Mota-Sanchez & Hollingworth R.M. eds. Global pesticide resistance in arthropods. CAB International, Cambridge, MA.

[60] White GB, 1974. *Anopheles gambiae* complex and disease transmission in Africa. *Trans R Soc Trop Med Hyg* 68:278-301.

[61] WHO, 2006. Pesticides and their application for the control of vectors and pests of public health importance.

[62] Wilkes TJ, Matola YG, Charlwood JD, 1996. *Anopheles rivulorum*, a vector of human malaria in Africa. *Med Vet Entomol* 10: 108-110.

[63] Wondji CS, Dabire KR, Tukur Z, Irving H, Djouaka D, Morgan JC, 2011. Identification and distribution of a GABA receptor mutation conferring dieldrin resistance in the malaria vector *Anopheles funestus* in Africa. *Insect Bioch Mol Biol* 41: 484-491

[64] World Health Organisation Test procedures for insecticides resistance monitoring in malaria vectors, bio-efficacy and persistence of insecticides treated surfaces. WHO/CDS/MAL, 1998, 12p

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The Role of *Anopheles gambiae* P450 Cytochrome in Insecticide Resistance and Infection

Rute Félix and Henrique Silveira
Centro de Malária e Outras Doenças Tropicais, UEI Parasitologia Médica, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa
Portugal

1. Introduction

Anopheles gambiae is the major vector of malaria transmission in sub-Saharan Africa where the disease is responsible for the highest morbidity and mortality worldwide. Malaria, nowadays, is still a major burden causing the death of nearly one million people each year, mostly children under the age of five, and affecting those living in the poorest countries (World Health Organization [WHO], 2010).

Currently, the major obstacles to malaria eradication are the absence of a protective vaccine, the spread of parasite resistance to anti-malarial drugs and the mosquito resistance to insecticides. Controlling mosquito vectors is fundamental to reduce mosquito-borne diseases. In fact, it has been one of the most used and effective method to prevent malaria, namely trough insecticides spraying and impregnated bed nets. These methods are highly dependent on a single class of insecticides, the pyrethroids, which are the most frequently used compounds for indoor residual spraying, and the only insecticide class used for insecticide treated nets (WHO, 2010). The extensive use of a single class of insecticides further increases the risk of mosquitoes developing resistance, which could rapidly lead to a major public health problem mainly in sub-Saharan countries where insecticidal vector control is being used widely (WHO, 2010). Strategies to control malaria are still not enough to totally eliminate malaria transmission, having yet to overcome several difficulties as the development of parasite drug resistance and mosquito-vector insecticide resistance (Yassine & Osta, 2010). Unfortunately the emergence of mosquito populations capable of withstanding insecticide exposure is threatening the efficiency of these control measures.

2. Insecticide resistance

Resistance has been defined as 'the inherited ability of a strain of some organisms to survive doses of a toxicant that would kill the majority of individuals in a normal population of the same species' (Scott, 1999). The evolution of insecticide-resistant mosquito strains is an increasing problem and one of the major obstacles for the control of medical and agricultural arthropod pests. Therefore, a better understanding of its genetic and biological basis is critical. Insecticide resistance can also lead to outbreaks of human diseases when

vectors cannot be controlled. Hence, the elucidation of resistance mechanisms is extremely important for the development of tools to monitor resistance in populations, thereby contributing to mosquito control programs. Although the mechanisms by which insecticides become less effective are similar across all vector taxa, each resistance problem is potentially unique and may involve a complex pattern of resistance *foci* (Brogdon & McAllister, 1998).
The main forms of resistance mechanisms can be divided in two groups: target site resistance, which occurs when the insecticide no longer binds to its target, and metabolic resistance, which occurs when enhanced levels of modified activities of detoxification enzymes prevent the insecticide from reaching its site of action. Alone or in combination these mechanisms confer resistance, sometimes at high levels, to all classes of insecticides.

2.1 Target site resistance

Target site resistance is based on alterations of amino acids in the site of action where the insecticide is supposed to bind, causing the insecticide to be less effective or ineffective at all. Knock down resistance (*Kdr*) occurs due to a single or multiple substitutions in the sodium channel (Martinez-Torres et al., 1998; Ranson et al., 2000a); and alteration in acetylcholinesterase results in decreased sensitivity to insecticides (Mutero et al., 1994). Insecticide resistance has been reported from many insects including *A. gambiae* that showed the presence of insensitive acetylcholinesterase in two different populations that were resistant to carbosulfan, a carbamate insecticide (N'Guessan et al., 2003). Mutations at a single codon in the *Rdl* (resistance to dieldrin) gene have been documented in all dieldrin-resistant insects, and confer both insensitivity to the insecticide and a decrease rate of desensitisation (ffrench-Constant et al., 1998). However, in *A. gambiae* this type of resistance mechanism has not been described so far. Those are examples of target site resistance that is not the object of the present review.

2.2 Metabolic resistance

Metabolic resistance usually involves over-expression of enzymes capable of detoxifying insecticides or modifications in the amino acid sequences that cause alterations in the levels and activity of detoxifying proteins. There are three major enzyme families involved in this type of resistance, glutathione-S-transferases (GST), carboxylesterases and P450 cytochromes. Carboxylesterases are mainly involved in organophosphate and carbamate and to a lesser extent in pyrethroid resistance, while P450 cytochromes are mainly involved in the metabolism of pyrethoids and to a lesser extent, detoxification of organophosphates and carbamates (Hemingway & Ranson, 2000). Glutathione S-transferases are involved in the detoxification of a wide range of xenobiotics, including the organochloride insecticide DDT (Enayati et al., 2005). In *A. gambiae* metabolic resistance to insecticides can be conferred by elevation in the activity of these three classes of detoxifying enzymes.
The over-expression of carboxylesterases as an evolutionary response to organophosphorus and carbamate insecticide selection pressure has been reported in several insects, including mosquitoes (Newcomb et al., 1997; Vulule et al., 1999; Zhu et al., 1999). Organophosphorus and carbamate inhibit B esterases by rapid esterification of the serine residue in the active site, usually followed by a slow hydrolysis of the new ester bond. Therefore, these insecticides can be considered as inhibitors of esterases, because they are poor substrates which have a high affinity for these enzymes (Hemingway & Karunaratne, 1998). Carboxylesterases in large amounts causes resistance as the insecticides are rapidly sequestered, even before reaching the target-site acetylcholinesterase (Hemingway &

Karunaratne, 1998). There are many reports of over expression of carboxylesterases in insecticide resistant mosquitoes including *A. gambiae*, where enhanced production of carboxylesterases was observed in permethrin-resistant mosquitoes (Vulule et al., 1999).
Glutathione S-transferases are a major class of detoxification enzymes that possess a wide range of substrates specificities (Enayati et al., 2005). Elevated GST activity has been implicated in resistance to several classes of insecticides (Ranson et al., 2001). Higher enzyme activity is usually due to an increase in the amount of one or more enzymes, either as a result of gene amplification or more commonly through increases in transcriptional rate, rather than qualitative changes in individual enzymes (Hemingway et al., 2004). The primary function of GSTs is the detoxification of both endogenous and xenobiotic compounds either directly or by catalysing the secondary metabolism of a vast array of compounds oxidised by P450 cytochromes (Wilce & Parker, 1994). GST enzymes metabolise insecticides by facilitating their reductive dehydrochlorination or by conjugation reactions with reduced glutathione to produce water soluble metabolites that are more readily excreted (Wilce & Parker, 1994). They also contribute to the removal of toxic oxygen free radical species produced through the action of pesticides (Enayati et al., 2005). In *A. gambiae* elevated GST levels were shown to be associated with DDT resistance (Ranson et al., 2001). Furthermore genetic mapping of the major *loci* conferring DDT resistance in *A. gambiae* implicate both *cis*- and *trans*-acting factors in the overexpression of GSTs (Ranson et al., 2000b). GSTs in *A. gambiae* were over expressed in a DDT-resistant strain, but only one *GSTE2-2* was able to metabolise DDT (Ortelli et al., 2003).
P450 cytochromes are a complex family of enzymes that are involved in the metabolism of xenobiotics and have a role in the endogenous metabolism. P450 cytochromes mediated resistance is probably the most frequent type of insecticide resistance. They are involved in the metabolism of virtually all insecticides, leading to activation of the molecule in the case of organophosphorus insecticides, or more generally to detoxification (Scott & Wen, 2001). In most cases where a link between insecticide resistance and elevated P450 activity has been shown, the P450 cytochrome belongs to the *CYP6* family (Nikou et al., 2003; Djouaka et al., 2008; Müller et al., 2007; McLaughlin et al., 2008). Although being difficult the identification of the specific P450 cytochrome associated with resistance, several P450 cytochromes were already isolated from insecticide resistant strains (Dunkov, et al., 1997; Kasai & Scott, 2000; Sabourault et al., 2001).

3. Insect P450 cytochromes

P450 Cytochromes are hemoproteins which act as terminal oxidases in monooxygenase systems. P450 cytochromes, whose name originated on its characteristic absorbance peak at 450 nm that appears when these enzymes are reduced and saturated with carbon-monoxide, constitute one of the oldest and largest super families of enzymes being found in almost all living organisms. In the literature, P450 enzymes are known by several names: cytochromes P450 monooxigenases, mixed functions oxidases, microsomal oxidases and heme thiolate proteins.
Insect P450s play a critical role in the metabolism of a wide variety of endogenous and exogenous compounds such as steroids, fatty acids and a wide range of xenobiotics and have also been implicated in vital processes like growth, development, feeding, reproduction, insecticide resistance and tolerance to plant toxins (Feyereisen, 1999; Scott et al., 1998; Scott, 1999). P450 cytochromes are also intimately involved in the synthesis and

degradation of insect hormones and pheromones, including 20-hydroxyecdysone and juvenile hormone (Feyereisen, 1999).

3.1 Nomenclature

To distinguish one of these cytochromes among all the P450s, a standardized nomenclature system was implemented (Nebert et al., 1991; Nelson et al., 1996). Each P450 is named with CYP, followed by an Arabical number for the gene family, a letter for the sub-family and another Arabical number for the gene. Cytochromes P450s with share more then 40% of the amino acids are usually grouped into the same family and members with >55% of the amino acids identical are normally grouped in the same sub-family. However, there are exceptions to these rules (Nelson et al., 1996). As it is based on amino acid similarities, no information regarding the function of each P450 should be assumed from its name.

3.2 Structure

P450s can be divided into classes depending on how electrons from NAD(P)H are delivered to the catalytic site. Class I P450s are found in eukaryotes and are associated with mitochondrial membranes. This class of enzymes requires both a FAD-containing reductase and an iron sulphur redoxin, and catalyzes several steps in the biosynthesis of steroid. Class II enzymes are the most common in eukaryotes and are found in the endoplasmic reticulum. These enzymes only require an FAD/FMN-containing P450 reductase for transfer of electrons. Their functions are extremely diverse and, in eukaryotes, include aspects of the biosynthesis and catabolism of signalling molecules and steroid hormones (Feyereisen, 1999). Class III enzymes are self-sufficient and require no electron donor. They are involved in the synthesis of signalling molecules. Finally, class IV enzymes receive electrons directly from NAD(P)H. Class I and II P450s from all organisms participate in the detoxification or sometimes the activation of xenobiotics and class III and IV enzymes are considered remains of the ancestral forms of P450s involved in detoxification of damaging activated oxygen species (Werck-Reichhart & Feyereisen, 2000).

Most P450s are approximately 500 amino acids long. The core of these proteins is formed by a four-helix bundle, two sets of β sheets, two helices and a coil called the "meander". A characteristic consensus sequence known as the P450 "signature" FXXGXXXCXG, located on the C-terminus of the heme binding region, contains a conserved cysteine that serves as a fifth ligand to the heme iron. There are two other conserved motifs specific of the P450 proteins. One is the DGXXT domain, which corresponds to the proton transfer groove on the distal site of the heme. Another is the EXXR domain, which is probably needed to stabilize the core structure located on the proximal side of heme (Werck-Reichhart & Feyereisen, 2000).

3.3 Microssomal / mitochondrial

In insects both mitochondrial and microssomal P450 systems have been described. The majority of P450 in insects are microssomal, located in the endoplasmic reticulum, and require the flavoprotein NADPH cytochrome P450 reductase as the main electron donor; however cytochrome b_5 is sometimes needed, depending of the substrate and of the P450 cytochrome involved. Mitochondrial P450 are also present, but, differently from microssomal P450, require ferridoxin and a NADPH ferridoxin reductase as electron donor (Scott & Wen, 2001).

3.4 Characterization / function

Cytochromes P450 enzymes catalyse thousands of different reactions, which are based on the activation of molecular oxygen, with insertion of one of its atoms into the substrate, and reduction of the other to form water (Guengerich, 1991). P450s use electrons from NAD(P)H to catalyse the activation of molecular oxygen, leading to the regiospecific and stereospecific oxidative attack of structurally diverse chemicals (Werck-Reichhart & Feyereisen, 2000).

The interaction that occurs between P450 cytochromes and the NADPH–cytochrome P450 reductase is better expressed as a cyclic reaction (Guengerich, 1991) as it is depicted in Figure 1.

The cycle is initiated by the binding of the substrate to the ferric form of the enzyme to form an enzyme-substrate complex, followed by a reduction of the ferric complex by an electron transferred from NADPH via NADPH-cytochrome P450 reductase. Next, the binding of molecular oxygen to the reduced complex forms an enzyme-oxygen-substrate complex followed by the transference of a second electron from NADPH via NADPH-cytochrome P450 reductase or from cytochrome b_5. A second proton is added, which results in the breaking of the oxygen-oxygen bond, releasing one atom of oxygen as water. The oxygen atom remaining is transferred to the substrate, originating an oxidized product, which is released, and a ferric form of the enzyme is once more generated. Then the cycle is re-initiated (Guengerich, 1991).

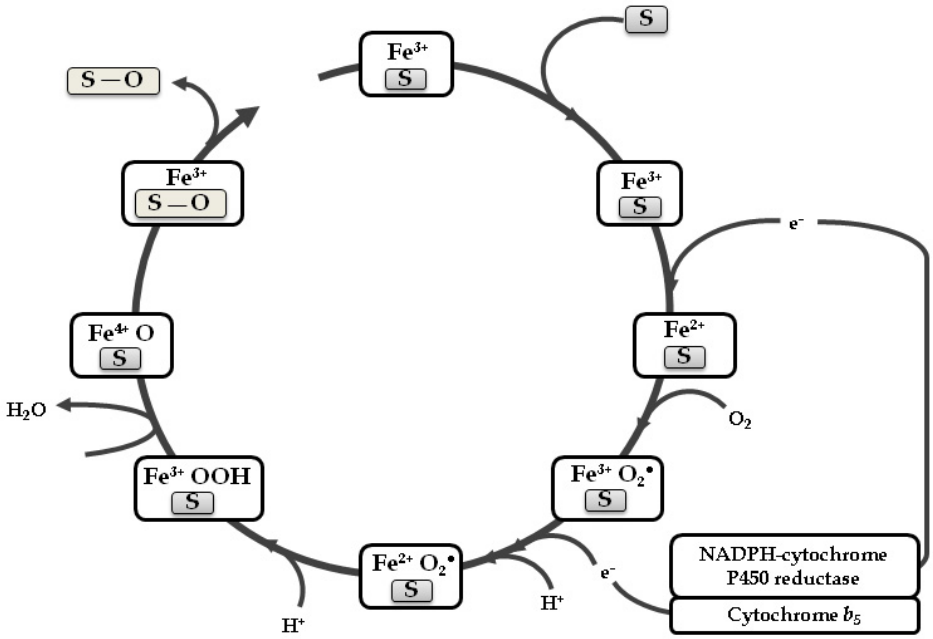

Fig. 1. Catalytic mechanism of P450 enzymes, where S is the substrate.

3.5 Diversity and specificity

The huge diversity of P450 cytochromes is probably due to an extensive process of gene duplication and cases of gene amplification, conversion, genome duplication, gene loss and lateral transfer (Werck-Reichhart & Feyereisen, 2000). Due to their extremely diverse functions, they can be found with different patterns of expression in all types of tissues and in almost all types of organisms. Although being expressed in a wide range of tissues, insect P450s have their highest activity associated with midgut, fat body and malpighian tubules (Feyereisen, 1999; Scott, 1999).

Additionally, P450s metabolise a large number of substrates, probably due to the existence of numerous P450 isoforms and to the broad specificity of some isoforms (Scott & Wen, 2001). Nevertheless the substrate specificity and type of reaction catalysed by each P450 cytochrome is still not well understood.

Their diversity enables individual P450 cytochromes to display different expression patterns related to life stages, tissues, inducers/inhibitors and substrates. There are P450s that are expressed in all life stages (*CYP12* genes) while others are only expressed in adults (*CYP6Z1*) or in larval stages (*CYP6Z3*) (Nikou et al., 2003). Although being found expressed in almost all types of tissues, there are P450s which are tissue specific, while others are expressed everywhere (Feyereisen, 1999; Scott et al., 1998; Scott & Wen, 2001). Expression of P450 cytochromes may also be sex specific, as some P450s showed higher levels of expression in males compared with females (Muller et al., 2007; Nikou et al., 2003).

A large variation in substrate specificity can also be found among different P450s, some being capable of metabolising several substrates while others have only one known substrate (Scott, 1999; Scott et al., 1998). There can be also some overlapping substrate specificity among P450 cytochromes, so that one compound could be metabolised by several enzymes. The production of one or several metabolites from a single substrate also differs depending on the P450s. P450s show a vast variation in response to inducers and inhibitors, each P450 can be induced/inhibited by one or several compounds. Some P450s can also remain unaltered while others are induced or repressed (Scott et al., 1998).

4. *Anopheles gambiae* P450 cytochromes and insecticide resistance

The *A. gambiae* genome has 111 annotated P450 cytochromes (Ranson et al., 2002). The great interest in these cytochromes derives from their role in the oxidative metabolism of insecticides, but only in few cases a definitive link between an increased expression of a specific P450 cytochrome and increased insecticide metabolism has been established.

Increasing reports of specific *A. gambiae* P450 cytochromes being involved in insecticide resistance have been published in the past. The involvement of P450s in pyrethroid resistance started to be demonstrated in *A. gambiae* from Kenyan villages, in synergistic studies using specific P450 cytochrome inhibitors and also given the detection of increased heme levels in resistant mosquitoes (Vulule et al., 1999).

In 2003, Nikou et al., verified that a P450 cytochrome (*CYP6Z1*) was over-expressed in a pyrethroid-resistant strain of *A. gambiae,* and the development of her work pointed to an implication of the involvement of this P450 in conferring pyrethroid resistance to this mosquito (Nikou et al., 2003).

Later, a microarray *chip* was constructed containing fragments from 230 genes associated with detoxification (David et al., 2005) to further study the metabolic based insecticide resistance in *A. gambiae*. From this work resulted the identification of, among other genes,

several P450 cytochromes that were highly expressed in the *A. gambiae* permethrin or DDT-resistant strains (David et al., 2005). Of notice is the P450 cytochrome *CYP325A3,* which belongs to a class that was not associated with insecticide resistance before and which was highly over-expressed in an *A. gambiae* permethrin resistant strain. Additionally, *CYP325A3* was later reported as constitutively over-expressed in a Nigerian pyrethroid resistant strain of *A. gambiae* (Awolola et al., 2009).

In 2007, studies regarding a recently colonised strain of *A. gambiae* from Ghana identified genes whose expression levels were associated with pyrethroid resistance. Among these were three P450 cytochromes (*CYP6M2, CYP6Z2* and *CYP6Z3*) (Muller et al., 2007). These results, together with their location within a cluster of P450 cytochromes in the right arm of chromosome 3 (3R), which is in close association with a pyrethroid resistance QTL (Ranson et al., 2004), strongly support their involvement in insecticide resistance. A subsequent study showed that *CYP6Z2* displays broad substrate specificity, which may be associated with xenobiotics metabolism and detoxification (Mclaughlin et al., 2008). Despite, *CYP6Z2* being able to bind to permethrin and cypermethrin, *CYP6Z2* does not metabolise neither one of these insecticides (Mclaughlin et al., 2008).

In 2008, Djouaka et al. also identified several P450 cytochromes over-expressed in one or more pyrethroid resistant populations of *A. gambiae.* Among these were *CYP6P3* and once again *CYP6M2*. Both genes showed high levels of over-expression in all the resistant populations, but the first was the gene that showed greatest differences. In the same year, *CYP6P3* was also identified as being up-regulated in another highly permethrin resistant *A. gambiae* population (Müller et al., 2008).

Recent studies on *A. gambiae* recombinant proteins *CYP6M2* (Stevenson et al., 2011) and *CYP6P3* (Müller et al., 2008) demonstrated that these enzymes could metabolise pyrethroids. Thus, the up regulation of these P450 cytochromes in pyrethroid resistant populations, strongly supports a key role for these genes to confer pyrethroid resistance in *A. gambiae*.

Highly expressed P450s have been also reported in DDT resistant strains of *A. gambiae* (David et al., 2005). *CYP6Z1* and *CYP12F1* were strongly over-expressed together with other genes, suggesting that multiple genes could contribute to the DDT resistance phenotype. The slightly over-expression of the electron donor cytochrome P450 reductase in the DDT resistant strain further supported a P450-based resistance mechanism in *A. gambiae* (David et al., 2005).

As the above P450 cytochromes, *CYP314A1* was also found to be over-expressed in a DDT resistant strain of *A. gambiae* from Kenia (Vontas et al., 2005), suggesting a possible involvement in the insecticide resistance phenotype. Both *CYP6Z1* and *CYP6Z2* were over-expressed in DDT resistant strains of *A. gambiae* (David et al. 2005). Although being very similar, these two cytochromes have predicted substrate cavities dramatically different and *CYP6Z1* was predicted to be the only one capable of metabolizing DDT. Chiu et al. (2008) through biochemical characterisations supported these predictions and identified *CYP6Z1* as the only P450 cytochrome capable of metabolising DDT, demonstrating its potential as a target to reduce *A. gambiae* resistance to DDT (Chiu et al., 2008).

Another evidence of the involvement of P450s in insecticide resistance is the fact that silencing the main electron donor of P450 cytochromes, the cytochrome P450 reductase, by RNAi, greatly increased the susceptibility of *A. gambiae* to permethrin, emphasising the important chemoprotective role of P450 cytochromes in this process (Lycett et al., 2006).

Nevertheless, although P450s have been clearly associated with insecticide resistance, the identification of specific P450 cytochromes responsible for insecticide resistance is still extremely difficult.

5. *Anopheles gambiae* P450 cytochromes and malaria infection

P450 cytochromes have also been implicated in other vital processes as in *A. gambiae* response to bacterial challenge and to parasite invasion, but the real importance and function of these cytochromes in this process is still not well understood.

A genome expression analysis of *A. gambiae* was made to identify which genes responded to injury, bacterial challenge and malaria infection (Dimopoulos et al., 2002). This study identified three P450 cytochromes, one associated with injury, microbial challenge and oxidative stress; the second associated with the response to septic injury which is similar to a bacterial infection *in vivo*; and the third associated with the response to malaria infection and the presence of lipopolysaccharide (Dimopoulos et al., 2002).

The involvement of P450 cytochromes in response to microbial challenge was established when two P450 cytochromes (*CYP4C27* and *CYP306A1*) were differently expressed in the presence of Gram negative (*Salmonella thyphimurium*) or Gram positive (*Staphylococcus aureus*) bacteria (Aguilar et al., 2005). This involvement was even more evident when a study, trying to implicate the mosquito midgut microbiota in the defense against malaria parasites, showed that there were ten P450s differently expressed in response to *Escherichia coli* and *S. aureus* in the *A. gambiae* midgut twelve hours after an uninfected blood meal (Dong et al., 2009). Between the P450 cytochromes differently expressed there were *CYP4H17, CYP6M3, CYP6AG1, CYP9J5*, two of them were mitochondrial cytochromes, *CYP49A1* and *CYP12F4* (Dong et al., 2009).

Regarding the relation between P450 cytochromes and the response to malaria infection, it was partly unveiled for the first time in a study about the midgut epithelial responses to *Plasmodium* invasion (Vlachou et al., 2005). The study revealed that P450 cytochromes were differentially expressed during different phases of the midgut invasion (before invasion, during invasion and after invasion) as well as when they compared *Plasmodium* wild-type infection with *Plasmodium* that were unable to invade the epithelium. (Vlachou et al., 2005). P450s that stood out in this study were *CYP305A1, CYP304B1, CYP6Z1* and *CYP6M4* (Vlachou et al., 2005). The role of P450 cytochromes in the *A. gambiae* response to malaria infection has been reinforced in the last years. Comparing the *A. gambiae* response to two different *Plasmodium* parasites -*P. berghei* and *Plasmodium falciparum* - showed that the mosquito induced slightly different immune responses to each parasite, and that the mosquito was capable of sensing infected blood constituents and mount an immune response, even in the absence of invading ookinetes (Dong et al., 2006). Although there were different responses between the three experimental groups, in all of them there were P450s differentially expressed in the midgut (*CYP6AG1, CYP6M4, CYP6M1, CYP9J5* and *CYP12F3*) and in the fat body (*CYP6AG1* and *CYP4G17*), reinforcing, their involvement in response to malaria infection.

Further evidence of the link between P450 cytochromes and the mosquito's response to malaria infection came from different studies. First, the effect on gene regulation of the presence of chloroquine in an uninfected blood meal and in a *Plasmodium* infected blood meal was investigated (Abrantes et al., 2008). This work showed that chloroquine affects the abundance of transcripts which encode proteins involved in a variety of processes,

including P450 cytochromes that were differently expressed in the *P. berghei* infected blood meal (*CYP9L1, CYP304B1* and *CYP305A1*). A second study focused on the role of *A. gambiae* detoxification enzymes, from the three major families involved in detoxification, GSTs, carboxylesterases and P450 cytochromes, in the response to *Plasmodium* infection (Félix et al., 2010). In this study the impact of *P. berghei* infection was analyzed at two time points: one day following the blood meal, during which parasites invade the midgut epithelium, and eleven days after the blood meal when sporozoites were starting to be released to the hemolymph; in two different tissues, midgut and fat body. At day one after the *Plasmodium* infected blood meal they found 17 P450 cytochromes down-regulated and 5 P450 cytochromes up-regulated, including *CYP9L1, CYP304B1, CYP325H1, CYP6M2* and *CYP6Z2* in the midgut, and 5 P450 up-regulated and 1 down-regulated in the fat body, including *CYP12F2, CYP6M2, CYP6M3* and *CYP4G17*. At eleven days after an infected blood meal they found 2 P450 cytochromes up-regulated and 3 down-regulated in the midgut and 1 P450 cytochrome up-regulated and 1 down-regulated in the fat body. The high number of P450 cytochromes differently expressed by the presence of *P. berghei* parasites in different phases of infection and in different tissues suggests that P450 cytochromes are deeply involved in the mosquito response to *Plasmodium* infection, having an important role in different development stages of the parasite and covering different tissues of the mosquito. More specifically, these P450 cytochromes might have a direct role in *Plasmodium* response during the parasite invasion of the midgut epithelium as this is the moment and tissue where more P450 were differentially expressed. The over expression of these P450 cytochromes could be part of a mosquito response mechanism to parasite invasion occurring in the midgut. One possibility is that P450s are involved in the cytoskeleton rearrangement (Vlachou et al., 2005; Vlachou & Kafatos, 2005), or alternatively P450s could be involved in the production of nitric oxide and other reactive oxygen radicals that are induced by *Plasmodium* invasion of the midgut epithelium (Han et al., 2000; Luckhart et al., 1998). The blood meal *per si* generates metabolic changes that are also expected to increase the oxidative stress in the mosquito midgut, which is augmented by the presence of *Plasmodium* parasites (Molina-Cruz et al., 2008). Moreover, other parasite killing mechanisms also induce oxidative stress inside the host which, although helping to eliminate the parasite, are also toxic to the host cell. The high level of oxidative stress inside the host cell could trigger cellular and molecular regulation of these P450 cytochromes, at this time point, being responsible for host detoxification and parasite elimination.

Mosquito hemocytes mediate important cellular immune responses including phagocythosis, encapsulation and secrete immune factors such as antimicrobial peptides and mediate melanization. Recently, studies were made to characterize the role of *A. gambiae* hemocytes in mosquito immunity, consisting in a genome-wide transcriptomic analysis of adult female hemocytes following infection by bacteria and *Plasmodium* parasites (Baton et al., 2009). This work showed that *CYP325H1* and *CYP6M1* were differently expressed in the presence of *Micrococcus luteus*, a Gram-positive bacteria (Baton et al., 2009), reinforcing the role of P450 cytochromes in response to microbial challenge. This work also showed that *CYP325H1* was differently expressed 24 hours after the infected blood meal, during *P. berghei* ookinete invasion of the midgut epithelium. Moreover, *CYP6AG1* and *CYP6M3* were also differentially expressed 19 days after the infected blood meal, during *P. berghei* sporozoite migration through the hemolymph (Baton et al., 2009), suggesting that P450 cytochromes have a role in the response to malaria infection by hemocytes. Another study aiming to analyze the transcriptional profile of circulating *A. gambiae* hemocytes during *P.*

berghei infection showed that *CYP6Z1*, *CYP6M2*, *CYP6M3* and *CYP12F2* were differently expressed at 24-28 hours after an infective blood meal (Pinto et al., 2009), valuing the importance of P450 cytochromes on the hemocyte response to malaria parasite invasion.

6. Conclusion

The role of P450 cytochromes during *Plasmodium* invasion is still poorly understood, but it may play out to be of utmost importance to combat malaria transmission. Here, we intend to bring an update review on the connection between P450 cytochromes and the *A. gambiae* response to malaria infection, identifying several P450 cytochromes that probably are, directly or indirectly, involved in the response to *Plasmodium* invasion. We have also reviewed the implication of P450 cytochromes in *A. gambiae* insecticide resistance. However, uncovering the objective role of these cytochromes in insecticide resistance, that is naming specific cytochromes and describing in detail the processes in which those specific P450s are involved is still extremely difficult.

The consistent detection of differential expression of P450 cytochromes, in studies about either insecticide resistance or the response to malaria infection, suggests that the role of these P450s could be similar in these two processes. Nevertheless, the real importance and function of P450 cytochromes in these processes is still not well understood neither the possibility of interplay between infection and insecticide resistance. One of the P450 cytochromes with expression altered in response to insecticides and *Plasmodium* infection was *CYP6M2* that, was highly over-expressed in a pyrethroid-resistant strain of *A. gambiae* mosquitoes (Muller et al., 2007) and also highly over-expressed in response to *Plasmodium* infection in both the midgut and the fat body 1 day after an infected blood meal (Félix et al., 2010). These results suggest that the role of *CYP6M2* might be the same in response to insecticides and infection, or that these two processes might share the activation mechanism of *CYP6M2* expression. *CYP6M2* could also function as an endogenous mediator, acting as the first response to different challenges, which would explain being increased by parasite infection and insecticide exposure. Similar to *CYP6M2* is *CYP6Z1*, yet another P450 cytochrome that was over-expressed in insecticides-resistant strains of *A. gambiae* (David et al., 2005; Nikou et al., 2003) and was also over-expressed in response to *Plasmodium* infection (Vlachou et al., 2005). The increase in the expression of this P450 could function as an immediate response to an exogenous challenge or *A. gambiae* could have the same mechanism of response, including over-expression of specific P450 cytochromes, to parasite infection and insecticide exposure. *CYP6Z2* was highly over-expressed in a pyrethroid-resistant strain (Müller et al., 2007), but opposite to *CYP6M2*, was down-regulated in the midgut of *A. gambiae* at day 1 and day 11 after an infected blood meal (Félix et al., 2010). These results suggest a different role for *CYP6Z2* in response to the insecticide and to parasite infection, however, we have to take into account that, although being able to bind to permethrin and cypermethrin, *CYP6Z2* does not metabolise these compounds (Mclaughlin et al., 2008). So the over-expression of *CYP6Z2* in a pyrethroid-resistant strain might be associated with different processes other than insecticide resistance.

A more complete knowledge about the factors involved in P450 cytochromes response to malaria infection and insecticide resistance is extremely needed for the implementation of efficient malaria and vector control programmes, including strategies able to adapt to different types of resistance. Although the interaction of insecticides with P450 enzymes has been studied, many of its aspects still remains poorly understood. Grasping the underlying

processes in this interaction might help mitigate the problem of insecticide resistance, and therefore contribute to the control of malaria and other human diseases.

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8. References

Abrantes, P.; Dimopoulos, G.; Grosso, A.R.; do Rosário, V.E. & Silveira, H. (2008). Chloroquine mediated modulation of *Anopheles gambiae* gene expression. *PLoS One*, 3(7), e2587, ISSN 1932-6203

Aguilar, R.; Dong, Y.; Warr, E. & Dimopoulos, G. (2005). *Anopheles* infection responses; laboratory models versus field malaria transmission systems. *Acta Tropica*, 95(3), 285-291, ISSN 0001-706X

Awolola,T.S.; Oduola, O.A.; Strode, C.; Koekemoer, L.L.; Brooke, B. & Ranson, H. (2009). Evidence of multiple pyrethroid resistance mechanisms in the malaria vector *Anopheles gambiae* sensu stricto from Nigeria. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 103, 1139-1145, ISSN 0035-9203

Baton, L.A.; Robertson, A.; Warr, E.; Strand, M.R. & Dimopoulos, G. (2009). Genome-wide transcriptomic profiling of *Anopheles gambiae* hemocytes reveals pathogen-specific signatures upon bacterial challenge and *Plasmodium berghei* infection. *BMC Genomics*, 5, 10:257, ISSN 1471-2164

Brogdon, W.G. & McAllister, J.C. (1998). Insecticide resistance and vector control. *Emerging Infectious Diseases,* 4(4), 605-613, ISSN: 1080-6059

Chiu, T.L.; Wen, Z.; Rupasinghe, S.G. & Schuler, M.A. (2008). Comparative molecular modeling of *Anopheles gambiae* CYP6Z1, a mosquito P450 capable of metabolizing DDT. *Proceedings of the National Academy of Sciences*, 105(26), 8855-8860, ISSN 0027-8424

David, J.P.; Strode, C.; Vontas, J.; Nikou, D.; Vaughan, A.; Pignatelli, P.M.; Louis, C.; Hemingway, J. & Ranson, H. (2005). The *Anopheles gambiae* detoxification chip: a highly specific microarray to study metabolic-based insecticide resistance in malaria vectors. *Proceedings of the National Academy of Sciences*, 102(11), 4080-4084, ISSN 0027-8424

Dimopoulos, G.; Christophides, G.K.; Meister, S.; Schultz, J.; White, K.P.; Barillas-Mury, C. & Kafatos, F.C. (2002). Genome expression analysis of *Anopheles gambiae*: responses to injury, bacterial challenge, and malaria infection. *Proceedings of the National Academy of Sciences*, 99(13), 8814-8819, ISSN 0027-8424

Djouaka, R.F.; Bakare, A.A.; Coulibaly, O.N.; Akogbeto, M.C.; Ranson, H.; Hemingway, J. & Strode, C. (2008). Expression of the cytochrome P450s, CYP6P3 and CYP6M2 are significantly elevated in multiple pyrethroid resistant populations of *Anopheles gambiae s.s.* from Southern Benin and Nigeria. *BMC genomics*, 9, 538, ISSN 1471-2164

Dong, Y.; Aguilar, R.; Xi, Z.; Warr, E.; Mongin, E. & Dimopoulos, G. (2006). *Anopheles gambiae* immune responses to human and rodent *Plasmodium* parasite species. *PLoS Pathogens*, 2(6), e52, ISSN 1553-7366

Dong, Y.; Manfredini, F. & Dimopoulos, G. (2009). Implication of the mosquito midgut microbiota in the defense against malaria parasites. *PLoS Pathogens*, 5(5), e1000423, ISSN 1553-7366

Dunkov, B.C.; Guzov, V.M.; Mocelin, G.; Shotkoski, F.; Brun, A.; Amichot, M.; Ffrench-Constant, R.H. & Feyereisen, R. (1997). The *Drosophila* Cytochrome P450 Gene Cyp6a2: Structure, Localization, Heterologous Expression, and Induction by Phenobarbital. DNA and Cell Biology, 16(11), 1345-1356, ISSN 1044-5498

Enayati, A.A.; Ranson, H. & Hemingway, J. (2005). Insect glutathione transferases and insecticide resistance. *Insect Molecular Biology*, 14(1), 3-8, ISSN 0962-1075

Félix, R.C.; Müller, P.; Ribeiro, V.; Ranson, H. & Silveira, H. (2010). *Plasmodium* infection alters *Anopheles gambiae* detoxification gene expression. *BMC genomics*, 11, 312, ISSN 1471-2164

Feyereisen, R. (1999). Insect P450 Enzymes. *Annual Review of Entomology*, 44, 507-533, ISSN 0066-4170

ffrench-Constant, R.H.; Pittendrigh, B.; Vaughan, A. & Anthony, N. (1998). Why are there so few resistance-associated mutations in insecticide target genes? *Phil.Trans. R. Soc. Lond. B*, 353, 1685-1693, ISSN 1471-2970

Guengerich, F.P. (1991). Reactions and Significance of Cytochrome P-450 Enzymes. *The Journal of Biological Chemistry*, 266(16), 10019-10022, ISSN 0021–9258

Han, Y.S.; Thompson, J.; Kafatos, F.C. & Barillas-Mury, C. (2000). Molecular interactions between *Anopheles stephensi* midgut cells and *Plasmodium berghei*: the time bomb theory of ookinete invasion of mosquitoes. *Embo Journal*, 19(22), 6030-6040, ISSN 0261-4189

Hemingway, J. & Karunaratne, S.H. (1998). Mosquito carboxylesterases: a review of the molecular biology and biochemistry of a major insecticide resistance mechanism. *Medical and veterinary Entomology*, 12(1), 1-12, ISSN 1365-2915

Hemingway, J. & Ranson, H. (2000). Insecticide resistance in insect vectors of human disease. *Annual Review of Entomology*. 41, 371-391, ISSN 0066.4170

Hemingway, J.; Hawkes, N.J.; McCarrol, L. & Ranson, H. (2004). The molecular basis of insecticide resistance in mosquitoes. *Insect Biochemistry and Molecular Biology*, 34, 653-655, ISSN 0965-1748

Kumar, S.; Christophides, G.K.; Cantera, R.; Charles, B.; Han, Y.S.; Meister, S.; Dimopoulos, G.; Kafatos, F.C. & Barillas-Mury, C. (2003). The role of reactive oxygen species on *Plasmodium* melanotic encapsulation in *Anopheles gambiae*. *Proceedings of the National Academy of Sciences*, 100(24), 14139-14144, ISSN 0027-8424

Kumar, S.; Gupta, L.; Han, Y.S. & Barillas-Mury, C. (2004). Inducible peroxidases mediate nitration of *anopheles* midgut cells undergoing apoptosis in response to *Plasmodium* invasion. *Journal of Biological Chemistry*, 279(51), 53475-53482, ISSN 0021-9258

Kasai, S. & Scott, J.G. (2000). Overexpression of Cytochrome P450 CYP6D1 Is Associated with Monooxygenase-Mediated Pyrethroid Resistance in House Flies from Georgia. *Pesticide Biochemistry and Physiology*, 68, 34-41, ISSN 0048-3575

Luckhart, S.; Vodovotz, Y.; Cui, L. & Rosenberg, R. (1998). The mosquito *Anopheles stephensi* limits malaria parasite development with inducible synthesis of nitric oxide. *Proceedings of the National Academy of Sciences,* 95(10), 5700-5705, ISSN 0027-8424

Lycett, G.J.; McLaughlin, L.A.; Ranson, H.; Hemingway, J.; Kafatos, F.C.; Loukeris, T.G. & Paine, M.J. (2006). Anopheles gambiae P450 reductase is highly expressed in oenocytes and in vivo knockdown increases permethrin susceptibility. *Insect Molecular Biology*, 15(3), 321-327, ISSN 0962-1075

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*, 7(2), 179-184, ISSN 0962-1075

McLaughlin,L.A.; Niazi, U.; Bibby, J.; David, J.P.; Vontas, J.; Hemingway, J.; Ranson, H.; Sutcliffe, M.J. & Paine, M.J. (2008). Characterization of inhibitors and substrates of *Anopheles gambiae* CYP6Z2. *Insect Molecular Biology*, 17(2), 125-135, ISSN 0962-1075

Molina-Cruz, A.; DeJong, R.J.; Charles, B.; Gupta, L.; Kumar, S., Jaramillo-Gutierrez, G. & Barillas-Mury, C. (2008) Reactive Oxygen Species Modulate *Anopheles gambiae* Immunity against Bacteria and *Plasmodium*. *The Journal of Biological Chemistry*, 283(6), 3217-3223, ISSN 0021–9258

Müller, P.; Donnelly, M.J. & Ranson, H. (2007). Transcription profiling of a recently colonised pyrethroid resistant *Anopheles gambiae* strain from Ghana. *BMC genomics*, 8, 36, ISSN 1471-2164

Müller,P.; Warr, E.; Stevenson, B.J.; Pignatelli, P.M.; Morgan, J.C.; Steven, A.; Yawson, A.E.; Mitchell, S.N.; Ranson, H.; Hemingway, J.; Paine. M.J. & Donnelly, M.J. (2008). Field-caught permethrin-resistant *Anopheles gambiae* overexpress CYP6P3, a P450 that metabolises pyrethroids. *PLoS Genetics*, 4(11), e1000286, ISSN 1553-7390

Mutero, A.; Pralavorio, M.; Bride, J.M. & Fournier, D. (1994). Resistance-associated point mutations in insecticide-insensitive acetylcholinesterase. *Proceedings of the National Academy of Sciences*, 91(13), 5922-5526, ISSN 0027-8424

Nebert, D.W.; Nelson, D.R.; Coon, M.J.; Estabrook, R.W.; Feyereisen, R.; Fujii-Kuriyama, Y.; Gonzalez, F.J.; Guengerich, F.P.; Gunsalus, I.C.; Johnson, E.F.; Loper, J.C.; Sato, R.; Waterman, M.R. & Waxman, D.J. (1991). The P450 Superfamily: Update on New Sequences, Gene Mapping, and Recommended Nomenclature. *DNA and Cell Biology,* 10(1), 1-14, ISSN 1044-5498

Nelson, D.R.; Koymans, L.; Kamataki, T.; Stegeman, J.J; Feyereisen, R.; Waxman, D.J.; Waterman, M.R.; Gotoh, O.; Coon, M.J.; Estabrook, R.W.; Gunsalus, I.C. & Nebert, D.W. (1996). P450 superfamily: update on new sequences, gene mapping,

accession numbers and nomenclature. *Pharmacogenetics*, 6(1), 1-42, ISSN 0960-314X

Newcomb, R.D.; Campbell, P.M.; Ollis, D.L.; Cheah, E.; Russell, R.J. & Oakeshott, J.G. (1997). A single amino acid substitution converts a carboxylesterase to an organophosphorus hydrolase and confers insecticide resistance on a blowfly. *Proceedings of the National Academy of Sciences*, 94(14), 7464-7468, ISSN 0027-8424

N'Guessan, R.; Darriet, F.; Guillet, P.; Carnevale, P.; Traore-Lamizana, M.; Corbel, V.; Koffi, A.A. & Chandre, F. (2003). Resistance to carbosulfan in *Anopheles gambiae* from Ivory Coast, based on reduced sensitivity of acetylcholinesterase. *Medical and veterinary Entomology*, 17(1), 19-25, ISSN 1365-2915

Nikou, D.; Ranson, H. & Hemingway, J. (2003). An adult-specific CYP6 P450 gene is overexpressed in a oyrethroid-resistant strain of the malaria vector, *Anopheles gambiae*. *Gene*, 318, 91-102, ISSN 0378-1119

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*. *Biochemical Journal*, 373, 957-963, ISSN 0264-6021

Peterson, T.M.; Gow, A.J. & Luckhart, S. (2007). Nitric oxide metabolites induced in *Anopheles stephensi* control malaria parasite infection. *Free Radical Biology & Medicine*, 42(1), 132-142, ISSN 0891-5849

Pinto, S.B.; Lombardo, F.; Koutsos, A.C.; Waterhouse, R.M.; McKay, K.; Chunju, A.; Ramakrishnan, C.; Kafatos, F.C. & Michel, K. (2009). Discovery of *Plasmodium* modulators by genome-wide analysis of circulating hemocytes in *Anopheles gambiae*. *Proceedings of the National Academy of Sciences,* 106(50), 21270-21275, ISSN 0027-8424

Ranson, H.; Jensen, B.; Vulule, J.M.; Wang, X.; Hemingway, J. & Collins, F.H. (2000a). Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan Anopheles gambiae associated with resistance to DDT and pyrethroids. Insect Molecular Biology, 9 (5), 491-497, ISSN 0962-1075

Ranson, H.; Jensen, B.; Wang, X.; Prapanthadara, L.; Hemingway, J. & Collins, F.H. (2000b). Genetic mapping of two loci affecting DDT resistance in the malaria vector *Anopheles gambiae*. *Insect Molecular Biology*, 9(5), 499-507, ISSN 0962-1075

Ranson, H.; Rossiter, L.; Ortelli, F.; Jensen, B.; Wang, X.; Roth, C.W.; Collins, F.H. & Hemingway, J. (2001). Identification of a novel class of insect glutathione S-transferases involved in resistance to DDT in the malaria vector *Anopheles gambiae*. *Biochemical Journal*, 359, 295-304, ISSN 0264-6021

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, ISSN 0036-8075

Ranson, H.; Paton, M.G.; Jensen, B.; McCarroll, L.; Vaughan, A.; Hogan, J.R.; Hemingway, J. & Collins, F.H. (2004). Genetic mapping of genes conferring permethrin resistance

in the malaria vector, *Anopheles gambiae*. *Insect Molecular Biology*, 13 (4), 379-386, ISSN 0962-1075

Sabourault, C.; Guzov, V.M.; Koener, J.F.; Claudianos, C.; Plapp. F.W.Jr. & Feyereisen, R. (2001). Overproduction of a P450 that metabolizes diazinon is linked to a loss-of-function in the chromosome 2 ali-esterase (MdalphaE7) gene in resistant house flies. *Insect Molecular Biology*, 10 (6), 609-618, ISSN 0962-1075

Scott, J.G.; Liu, N. & Wen, Z. (1998). Insect cytochromes P450: diversity, insecticide resistance and tolerance to plant toxins. *Comparative Biochemistry and Phisiology Part C*, 121(1-3), 147-155, ISSN 1532-0456

Scott, J.G. (1999). Cytochromes P450 and insecticide resistance. *Insect Biochemistry and Molecular Biology*, 29(9), 757-777, ISSN 0965-1748

Scott, J.G. & Wen, Z. (2001) Cytochromes P450 of insects: the tip of the iceberg. *Pest Management Science*, 57(10), 958-967, ISSN 1526-4998

Stevenson, B.J.; Bibby, J.; Pignatelli, P.; Muangnoicharoen, S.; O'Neill, P.M.; Lian, L.Y.; Müller, P.; Nikou, D.; Steven, A.; Hemingway, J.; Sutcliffe, M.J. & Paine, M.J. (2011). Cytochrome P450 6M2 from the malaria vector *Anopheles gambiae* metabolizes pyrethroids: Sequential metabolism of deltamethrin revealed. *Insect Biochemistry and Molecular Biology*, in press, ISSN 0965-1748

Vlachou, D. & Kafatos, F.C. (2005). The complex interplay between mosquito positive and negative regulators of *Plasmodium* development. *Current Opinion in Microbiology*, 8(4), 415-421, ISSN 1369-5274

Vlachou, D.; Schlegelmilch, T.; Christophides, G.K. & Kafatos, F.C. (2005). Functioal Genomic Analysis of Midgut Epithelial Responses in *Anopheles* during *Plasmodium* Invasion. *Current Biology*, 15(13), 1185-1195, ISSN 0960-9822

Vontas, J., Blass, C. Koutsos, A.C., David, J.-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 Molecular Biology*, 14(5), 509-521, ISSN 0962-1075

Vulule, J.M., Beach, R.F., Atieli, F.K., McAllister, J.C., Brogdon, W.G., Roberts, J.M., Mwangi, R.W. & Hawley, W.A. (1999). Elevated oxidase and esterase levels associated with permethrin tolerance in *Anopheles gambiae* from Kenyan villages using permethrin-impregnated nets. *Medical and veterinary Entomology*, 13(3), 239-244, ISSN 1365-2915

Werck-Reichhart, D. & Feyereisen, R. (2000). Cytochromes P450: a success history. *Genome Biology*, 1(6), 3003, ISSN 1759-6653

Wilce, M.C. & Parker, M.W. (1994). Structure and function of glutathione S-transferases. *Biochim Biophys Acta*, 1205, 1-18, ISSN 0006-3002

World Health Organization (2010). World Malaria Report 2010, ISBN 978 92 4 156410. Geneve.

Yassine H. & Osta M.A. (2010). *Anopheles gambiae* innate immunity. *Cellular Microbiology*, 12(1), 1-9, ISSN 1462-5814

Zhu, Y.C.; Dowdy, A.K. & Baker, J.E. (1999). Differential mRNA expression levels and gene sequences of a putative carboxylesterase-like enzyme from two strains of the parasitoid *Anisopteromalus calandrae* (Hymenoptera: Pteromalidae). *Insect Molecular Biology*, 29(5), 417-421, ISSN 0962-1075

24

Genetic Toxicological Profile of Carbofuran and Pirimicarb Carbamic Insecticides

Sonia Soloneski and Marcelo L. Larramendy
Faculty of Natural Sciences and Museum, National University of La Plata
Argentina

1. Introduction

It's well known that the pesticide usages in agriculture have led increase in food production worldwide. Although the benefits of conventional agricultural practices have been immense, they utilize levels of pesticides and fertilizers that can result in a negative impact on the environment (WHO, 1988). Only for the 2006-2007, the total world pesticide amount employed was approximately 5.2 billion pounds (www.epa.gov). Their application is still the most effective and accepted method for the plant and animal protection from a large number of pests, being the environment consequently and inevitably exposed to these chemicals. Herbicides accounted for the largest portion of total use, followed by other pesticides, like insecticides and fungicides (www.epa.gov). The goal in pesticide investigation and development is identifying the specificity of action of a pesticide toward the organisms it is supposed to kill (Cantelli-Forti et al., 1993). Only the target organisms should be affected by the application of the product. However, because pesticides are designed and selected for their biological activity, toxicity on non-target organisms frequently remains a significant potential risk (Cantelli-Forti et al., 1993). The benefits in using pesticides must be weighed against their deleterious effects on human health, biological interactions with non-target organisms, pesticide resistance and/or accumulation of these chemicals in the environment (WHO, 1988). Pesticides are high volume, widely used environmental chemicals and there is continuous debate concerning their probable role in both acute and chronic human health effects (Cantelli-Forti et al., 1993; Hodgson & Levi, 1996). Among the potential risk effects of agricultural chemicals, carcinogenesis is of special concern. The genetic toxicities of pesticides have been determined by numerous factors like their biological accumulation or degradation in the environment, their metabolism in humans, and their action in cellular components such as DNA, RNA and proteins (Shirasu, 1975). It seems essential the determination of the genotoxic risks of these pesticides before they are used in agriculture. Therefore, the carcinogenic and mutagenic potential of a large amount of pesticides has been the object of an extensive and wide investigation (WHO, 1990). These results have great predictive value for the carcinogenicity of several pesticides (IARC, 1987). The International Agency for Research on Cancer (IARC) has reviewed the potential carcinogenicity of a wide range of insecticides, fungicides, herbicides and other similar compounds. Fifty-six pesticides have been classified with carcinogenic potential in different laboratory animals (IARC, 2003). Among them, and as a brief example, chemicals compounds as phenoxy acid herbicides, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), lindane,

methoxychlor, toxaphene, and some organophosphates have been reported with a carcinogenic potential in human studies (IARC, 2003).
Numerous well known pesticides have been tested in a wide variety of mutagenicity as well as DNA, chromosomal, and cellular damage endpoints (IARC, 2003). Several investigations have been reported positive associations between exposure and pesticide risk (Shirasu, 1975; Bolognesi et al., 1993, 2009, 2011; Pavanello & Clonfero, 2000; Bolognesi, 2003; Clark & Snedeker, 2005; Castillo-Cadena et al., 2006).

2. Carbamic insecticides

The carbamates are chemicals mainly used in agriculture as insecticides, fungicides, herbicides, nematocides, and/or sprout inhibitors (IARC, 1976). These chemicals are part of the large group of synthetic pesticides that have been developed, produced, and used on a large scale within the last 50 years. Additionally, they are used as biocides for industrial or other applications as household products including gardens and homes (IARC, 1976).
During the last decades, considerable amounts of pesticides belonging to the class of carbamates have been released into the environment. Humans may be exposed to carbamates through food and drinking water around residences, schools, and commercial buildings, among others (IARC, 1976). Consequently, carbamates are potentially harmful to the health of different kinds of organisms (EPA, 2004). Among all classes of pesticides, carbamates are most commonly used compounds because organophosphates and organochlorines are extremely toxic and possess delayed neurotoxic effects (Hour et al., 1998). They share with organophosphates the ability to inhibit cholinesterase enzymes and therefore share similar symptomatology throughout acute and chronic exposures. Likewise, exposure can occur by several routes in the same individual due to multiple uses, and there is likely to be additive toxicity with simultaneous exposure to organophosphates (IARC, 1976).
The *N*-methyl carbamates are a group of closely related pesticides employed in homes, gardens and agriculture that may affect the functioning of the nervous system (EPA, 2007). Toxicological characteristics of the *N*-methyl carbamates involve maximal cholinesterase enzyme inhibition followed by a rapid recovery, typically from minutes to hours (EPA, 2007). Several compounds namely aldicarb, carbaryl, carbofuran, formetanate HCl, methiocarb, methomyl, oxamyl, pirimicarb, propoxur, and thiodicarb are included as members of the *N*-methyl carbamate class (EPA, 2007).

3. Carbofuran. Genotoxicity and cytotoxicity profiles

Carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate; CASRN: 1563-66-2) is one of the most widely granular employed *N*-methyl carbamate esters with both contact and systemic activity. Carbofuran is a derivative of carbamic acid being its chemical structure formula shown in Fig. 1.
Carbofuran is a relatively unstable compound that breaks down in the environment within weeks or months (www.inchem.org). It is registered on a variety of agricultural uses to control soil-dwelling and foliar-feeding insects, mites and nematodes on a variety of field, fruit, forage, grain, seed, and fiber crops (EPA, 2006). Carbofuran is a systemic, broad spectrum insecticide and nematocide registered *N*-methyl carbamate for control of soil and foliar pests. It has been reported for 2006 that nearly one million pounds of carbofuran was

applied worldwide (EPA, 2006). The most sensitive and appropriate effect associated with the use of carbofuran is its toxicity following acute exposure (HSDB, 2011). On the basis of its acute toxicity, it has been classified as a highly hazardous member (class Ib) by WHO (2009) and highly toxic compound (category I) by EPA (2006) based on its potency by the oral and inhalation exposure routes. In spite of the recommendation and regulation proposed by the United States Environmental Protection Agency (EPA) concerning the use of this carbamate within the United States of America, its application has been recently cancelled all over the Northern country by the same organization since 2009 (www.epa.gov). However, the contamination of environment with this compound can by far occur, particularly taking into consideration those countries where it is still in use and the probability of long-term low dose exposure becomes increased. Due to its extensive employment in agriculture and household, contamination of food, water and air has become serious and undesirable health problem for humans, animals and wildlife. Large quantities of this carbamate are particularly applied to different environments worldwide.

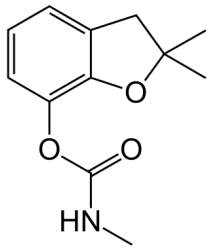

Fig. 1. Chemical structure of carborufan. Source: INCHEM (www.inchem.org).

Metabolism of carbofuran has been extensively studied in plants and animals (Dorough & Casida, 1964; Metcalf et al., 1968). In mammals, it reversibly inhibits acethylcholinesterase by carbamylation as well as others non-specific serine-containing enzymes, such as carboxylesterases and butyrylcholinesterases (Gupta, 1994). This results in accumulation of acetylcholine at nerve synapses and myoneural junctions leading to cholinergic signs and causing toxic effects (Karczmar, 1998). Epidemiological studies suggested that exposure to carbofuran may be associated with increased risk of gastrointestinal, neurological, cardiac dysfunction, and retinal degeneration (Cole et al., 1998; Kamel et al., 2000; Peter & Cherian, 2000). Carbofuran represents an acute poison when absorbed into the gastrointestinal tract by inhalation of dust and spray mist and minimally poison thought the intact skin contact (Gupta, 1994). In summary, carbofuran is reported to be teratogenic, embryotoxic and highly toxic to mammals (Gupta, 1994; WHO-FAO, 2004, 2009; WHO, 2009).

Genotoxicity and cytotoxicity studies have been conducted with this *N*-methyl carbamate member using several end-points on different cellular systems. A summary of the results reported so far is presented in Table 1.

The compound produced both conflicting and inconclusive results in mutagenicity tests varying according to either the end-point assessed (WHO, 1988, 2000-2002, 2009; WHO-FAO, 2004, 2009). When mutagenic activity was assessed in bacterial systems either positive or negative results have been reported. Carbofuran has been found to be non-mutagenic in *Salmonella typhimurium* since negative or weak positive response were observed in the number of mitotic recombinants regardless of the presence or absence of a rat liver

metabolic activation system (Blevins et al., 1977a; Gentile et al., 1982; Waters et al., 1982; Haworth & Lawlor, 1983; Hour et al., 1998; Yoon et al., 2001). These results indicate that carbofuran cannot be considered mutagenic in bacterial systems. However, it was active in *Salmonella typhimurium* TA1538 and TA98 strains in the presence or absence of S9 metabolic system (Gentile et al., 1982; Moriya et al., 1983; Hour et al., 1998). Whereas the insecticide did not induce reverse mutations in *Escherichia coli* (Simmon, 1979), it has been claimed as a relatively weaker mutagen with the repair defective Ames *Escherichia coli* K-12 test (Saxena et al., 1997). Similarly, positive results have been found after exposure in *Vibrio fischeri* regardless the absence or presence of S9 metabolic system (Canna-Michaelidou & Nicolaou, 1996). When DNA damage and repair assays were performed, carbofuran was also negative in both *Escherichia coli* and *Bacillus subtilis* bacterial systems (SRI, 1979). Similar negative results were also found after carbofuran exposure in *Saccharomyces cerevisiae* mitotic recombination assay (Simmon, 1979).

The mammalian *in vitro* gene mutation assay systems generated results consistent with the microbial gene mutation assays, although they were generally more responsive. When a mammalian cell system was employed for mutagenic screening, carbofuran was found to be positive in V79 cells (Wojciechowski et al., 1982). Similar results were reported for the cell mutation assay in mouse lymphoma L5178 cells (Kirby, 1983a, b). Unscheduled DNA synthesis was monitored in human fibroblasts and primary rat hepatocytes following treatment with the insecticide with and without S9 fraction. Both negative and positive results were obtained for the same endpoint in human primary fibroblasts regardless of the presence or absence of a rat liver metabolic activation system (Simmon, 1979; Gentile et al., 1982) but negative results were obtained in primary rat hepatocyte cultures (SRI, 1979). Single-strand breaks detected by alkaline comet assay were induced in *in vitro* human peripheral lymphocytes (Das et al., 2003; Naravaneni & Jamil, 2005). The induction of DNA fragmentation on human skin fibroblasts have been found to be enhanced after *in vitro* carbofuran treatment (Blevins et al., 1977b).

As opposed to mutation assays that detect specific gene defects, the chromosomal assays evaluate the structure of the whole chromosome. Five studies of carbofuran have evaluated the induction of sister chromatid exchanges in mammalian cell cultures. In one of the first studies, carbofuran was negative in Chinese hamster ovary cells regardless of the presence or absence of S9 fraction (Thilagar, 1983b). However, other authors reported positive results for the same cell system (Gentile et al., 1982; Thilagar, 1983c; Lin et al., 2007; Soloneski et al., 2008) as well as human lymphocytes (Georgian et al., 1985). Similarly, the effects on chromosomal structure following exposure to carbofuran were investigated in Chinese hamster ovary and primary human lymphocytes cells. While carbofuran did not induce *in vitro* chromosome damage in Chinese hamster ovary cells with or without metabolic system activation (Thilagar, 1983a), positive results were reported to occur not only in the same cellular system (Lin et al., 2007) but also in human lymphocytes *in vitro* (Pilinskaia & Stepanova, 1984; Das et al., 2003). However, inconclusive response for this endpoint has been also reported to occur in the latter system after carbofuran exposure (Naravaneni & Jamil, 2005). Positive results have been also reported for the ability of carbofuran to induce micronuclei in both Chinese hamster ovary cells and human lymphocytes *in vitro* with and without S9 metabolic fraction (Soloneski et al., 2008; Mladinic et al., 2009).

Several assays have been developed to assess the ability of carbofuran to cause cytotoxic effects on different cellular systems. Negative response was observed in both *Escherichia coli* and *Bacillus subtilis* bacterial systems (Simmon, 1979). When the analysis of cell-cycle

End-point/Test System	Concentration[a]	Results	References
In vitro assays			
Ames test			
Salmonella typhimurium, S9 +/-	100 – 10 000 μg/plate	+/-	Blevins et al., 1977a; Waters et al., 1982; Haworth & Lawlor, 1983; Yoon et al., 2001
Salmonella lactam assay, S9 +/-	1 – 10 000 μg/plate	-	Hour et al., 1998
	0.1 – 100 μg/plate	+	Gentile et al., 1982; Moriya et al., 1983; Hour et al., 1998
Pol A reverse mutation			
Escherichia coli (WP_2), S9 +/-	1 – 5 000 μg/plate	-	Simmon, 1979
Escherichia coli (K-12)	1 – 5 000 μg/plate	+	Saxena et al., 1997
Mutatox test			
Vibrio fischeri (M169), S9 +/-	175 μg/plate	+	Canna-Michaelidou & Nicolaou, 1996
DNA damage and repair			
Escherichia coli (W3110-p3478)	0 – 5 mg/6-mm disk	-	SRI, 1979
Bacillus subtilis (H17-M45)	0 – 5 mg/6-mm disk	-	SRI, 1979
Mitotic recombination			
Saccharomyces cerevisiae (D3), S9 +/-	1 – 50 mg/ml	-	Simmon, 1979
Gene mutation assay			
V79 cells	NA	+	Wojciechowski et al., 1982
Cell mutation tk locus			
Mouse lymphoma L5178 Y cells, S9 +/-	16 – 1 780 μg/ml	+/-	Kirby, 1983a, b
UDS	0.1 – 1 000 μg/ml	-	Simmon, 1979
Human fibroblasts (WI-38), S9 +/-	0.1 – 1 000 μg/ml	+	Gentile et al., 1982
Human lung fibroblasts	0 – 100 μg/ml	-	SRI, 1979
Primary rat hepatocytes			
Alkaline comet assay			
HL	NA	+	Naravaneni & Jamil, 2005
	0.5 – 4.0 μM	+	Das et al., 2003
DNA fragmentation analysis			
Human skin fibroblasts	NA	+	Blevins et al., 1977b
SCE assay			
CHO cells, S9 +/-	12.5 – 312.5 μg/ml	-	Thilagar, 1983b
CHO cells, S9 +/-	12.5 – 2 500 μg/ml	+	Thilagar, 1983c
CHO-K1 cells	5 – 100 μg/ml	+	Gentile et al., 1982; Soloneski et al., 2008
CHO-W8 cells	0.04 – 0.32 μg/ml	+	Lin et al., 2007
HL	NA	+	Georgian et al., 1985

Chromosomal aberrations			
CHO cells, S9 +/-	50 – 2 500 μg/ml	-	Thilagar, 1983a
CHO-W8 cells	0.04 – 0.32 μg/ml	+	Lin et al., 2007
HL	NA	+/-	Naravaneni & Jamil, 2005
HL	100 – 300 μg/ml	+	Pilinskaia & Stepanova, 1984
HL[b]	NA	+	Das et al., 2003
Micronuclei assay			
CHO-K1 cells	10 – 100 μg/ml	+	Soloneski et al., 2008
HL, S9 +/-	0.008 μg/ml	+	Mladinic et al., 2009
Growth inhibition			
Escherichia coli	1 – 500 mg/ml	-	Simmon, 1979
Bacillus subtilis	1 – 500 mg/ml	-	Simmon, 1979
Alteration in CCP			
CHO-W8 cells	0.04 – 0.32 μg/ml	-	Lin et al., 2007
CHO-K1 cells	50 – 100 μg/ml	+	Soloneski et al., 2008
CHL cells	30 μM	-	Yoon et al., 2001
Brain tubulin assembly assay			
Porcine cells	100 – 2 000 μmol/l	+	Stehrer-Schmid & Wolf, 1995
Cell viability			
CHL cells	30 μM	-	Yoon et al., 2001
CHO-K1 cells	50 – 100 μg/ml	+	Soloneski et al., 2008
Apoptosis			
CHL cells	30 μM	-	Yoon et al., 2001
Mouse brain microvascular endothelial cells	3 – 30 μM	-	Jung et al., 2003
Rat cortical cells	500 μM	+	Kim et al., 2004
In vivo assays			
Reverse mutation			
Zea mays	NA	-	Gentile et al., 1982
Sex-linked recessive lethal test			
Drosophila melanogaster	0 – 10 ppm	-	DeGraff, 1983; Gee, 1983
Dominant –lethal mutagenicity			
Mice	0.025 – 0.5 mg/Kg/day	-	FMC, 1971
UDS			
Rat hepatocytes	5 – 10 ppm	-	Valencia, 1981; 1983
Alkaline comet assay			
Mouse peripheral lymphocytes	0.1 – 0.4 mg/Kg bw	-	Zhou et al., 2005
HL[*]	NA	+	Castillo-Cadena et al., 2006

SCE			
Mouse peripheral lymphocytes	NA	+	Gentile et al., 1982
Rat	NA	+	Aly, 1998
Chromosomal aberrations			
Allium cepa	20 – 80 ppm	+	Saxena et al., 2010
Allium sativum	20 – 80 ppm	+	Saxena et al., 2010
Drosophila melanogaster	NA	-	Woodruff et al., 1983
Mouse bone marrow cells	3.8 – 1.9 (for 4 days) mg/Kg bw	+	Chauhan et al., 2000
Mouse bone marrow cells	0.1 – 1.0 mg/Kg bw	-	Pilinskaia & Stepanova, 1984
Rat bone marrow cells	0.6 – 10 mg/Kg bw	-	Putman, 1983b, a
HL[b]	NA	+	Zeljezic et al., 2009
Micronuclei			
Mouse peripheral lymphocytes	0.1 – 0.4 mg/Kg bw	-	Zhou et al., 2005
Mouse bone marrow cells	5.7 – 1.9 (for 4 days)	-	Chauhan et al., 2000
Alteration in CCP			
Allium cepa	20 – 80 ppm	+	Saxena et al., 2010

UDS, unscheduled DNA synthesis; SCE, sister chromatid exchange; HL, human lymphocytes; CCP, cell-cycle proliferation; NA, data not available.

Table 1. Evaluation of carbofuran-induced genotoxicity and cytotoxicity on different target systems. [a], expressed as reported by authors; [b], exposed to pesticide mixture containing carbofuran; [*], from agricultural workers occupationally exposed to carbofuran.

progression on mammalian cells was studied, carbofuran gave negative results in Chinese hamster ovary cells and lung fibroblasts (Yoon et al., 2001; Lin et al., 2007). On the other hand, Soloneski and co-workers (2008) reported a delay in the cell-cycle progression of Chinese hamster ovary cells after the insecticide treatment. Carbofuran was tested *in vitro* in the porcine brain tubulin assembly assay for detecting whether the chemical can be considered as a microtubule poison and an aneuploidy agent. A dose-dependent reduction in the degree of polymerization of tubulins was reported in porcine cells after *in vitro* treatment (Stehrer-Schmid & Wolf, 1995). Controversial results were reported for the cell viability assay in mammalian cells, e.g., Chinese hamster lung and ovary cells after the exposure (Yoon et al., 2001; Soloneski et al., 2008). Finally, whereas carbofuran-induced apoptosis has been reported in rat cortical cells (Kim et al., 2004), negative results have been also observed in mouse brain microvascular entothelial cells and Chinese lung fibroblasts (Yoon et al., 2001; Jung et al., 2003).
Similar end-points for both genotoxicity and cytotoxicity were also applied in *in vivo* systems. Carbofuran has been reported as a non inducer agent of mutations in plants cells, at least in *Zea mays* (Gentile et al., 1982), in the *Drosophila melanogaster* sex-linked recessive lethal test (DeGraff, 1983; Gee, 1983), and in the mice dominant-lethal mutagenicity test (FMC, 1971). Negative results have been obtained for the induction of unscheduled DNA synthesis in primary rat hepatocytes (Valencia, 1981, 1983). Controversial observations have been reported for the induction of DNA single-strand breaks assayed by the alkaline comet assay. Positive results were reported in circulating erythrocytes from occupationally exposed workers (Castillo-Cadena et al., 2006) whereas no induction was observed in mouse peripheral lymphocytes exposed *in vivo* (Zhou et al., 2005). It should be noted that the former positive

results could not be totally committed to carbofuran but to other pesticides, since the cohort of donors included in the study was exposed to a panel of other pesticides. Several reports were able to revealed that carbofuran increased the frequency of sister chromatid exchanges in mammalian cells from mouse and rats exposed *in vivo* (Gentile et al., 1982; Aly, 1998), and chromosomal aberrations in plants from *Allium* (Saxena et al., 2010), and mammals including occupationally exposed workers (Putman, 1983a, b; Pilinskaia & Stepanova, 1984; Chauhan et al., 2000; Zeljezic et al., 2009) but not in insects (Woodruff et al., 1983) as well as in rodent cells (Putman, 1983a, b; Pilinskaia & Stepanova, 1984). When the micronuclei induction end-point was employed in mouse, no induction was found either in bone marrow cells (Chauhan et al., 2000) or circulating lymphocytes (Zhou et al., 2005). Finally, alterations in the progression of the cell-cycle were reported to occur after carbofuran exposure in plants when the *Allium cepa* model was employed (Saxena et al., 2010).

4. Pirimicarb. Genotoxicity and cytotoxicity profiles

Pirimicarb (2-dimethylamino-5,6-dimethylpyrimidin-4-yl dimethylcarbamate, CASRN: 23103-98-2) is a dimethylcarbamate insecticide member with both contact and systemic activity. Similar to carbofuran, pirimicarb is a derivative of carbamic acid being its chemical structure formula shown in Fig. 2.

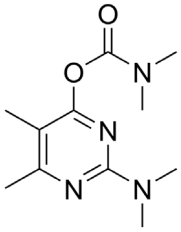

Fig. 2. Chemical structure of pirimicarb. Source: INCHEM (www.inchem.org)

Based on its acute toxicity, pirimicarb has been classified as a moderately hazardous compound (class II) by WHO (http://www.who.int/ipcs/publications/pesticides hazard/en/) and slightly to moderately toxic (category II-III) by EPA (1974a). Among carbamate pesticides, pirimicarb is registered as a fast-acting selective aphicide mostly used in a broad range of crops, including cereals, sugar beet, potatoes, fruit, and vegetables, and is relatively non-toxic to beneficial predators, parasites, and bees (WHO-FAO, 2004, 2009). It acts by contact, translaminar, vapor, and systemic action. Its mode of action is inhibiting acetylcholinesterase activity (WHO-FAO, 2004, 2009).

Available information on the genotoxic and cytotoxic properties of pirimicarb is limited and inconsistent. Only few data are available in the literature (WHO-FAO, 2004, 2009). Genotoxicity and cytotoxicity studies have been conducted with this carbamate using several end-points on different cellular systems. A summary of the results reported so far is presented in Table 2.

Pirimicarb has been generally recognized as non-genotoxic in bacteria, yeast and fungi as well as in mammalian cells (EPA, 1974b). It has been reported to be non-mutagenic in *Salmonella typhimurium* when the Ames reversion mutagenicity test for the TA1535, TA1538, TA98, and TA100 strains after S9 metabolic activation has been used (Trueman, 1980; Callander, 1995). Furthermore, similar situation was observed in both *Escherichia coli* and *Aspergillus nidulans* when the reverse mutation assay or recessive lethal gene mutation test

End-point/System	Concentration[a]	Results	References
In vitro assays			
Ames test			
Salmonella typhimurium, S9 +/-	2 500 µg/plate	-	Trueman, 1980
	5 000 µg/plate	-	Callander, 1995
Pol A reverse mutation			
Escherichia coli	5 000 µg/plate	-	Callander, 1995
Recessive lethal gene mutation			
Aspergillus nidulans	NA	-	Käfer et al., 1982
Cell mutation tk locus			
Mouse lymphoma L5178 Y cells	1 400 mg/ml – S9	-	Clay, 1996
	100 mg/ml + S9	+	Clay, 1996
Alkaline comet			
HL	50 – 500 µg/ml	+	Ündeger & Basaran, 2005
SCE assay			
CHO-K1 cells	100 – 200 µg/ml	+	Soloneski & Larramendy, 2010
Chromosomal aberrations			
CHO-K1 cells	10 – 300 µg/ml	+	Soloneski & Larramendy, 2010
HL, S9 +/-	500 µg/ml	-	Wildgoose et al., 1987
Alteration in CCP			
CHO-K1 cells	100 – 300 µg/ml	+	Soloneski & Larramendy, 2010
In vivo assays			
Eye mosaic system w/w+			
Drosophila melanogaster	NA	+	Aguirrezabalaga et al., 1994
Dominant lethal mutation			
Mice	20 mg/Kg bw	-	McGregor, 1974
UDS			
Rat liver cells	200 mg/Kg bw	-	Kennelly, 1990
Chromosomal aberrations			
Rat bone marrow cells	50/–100 mg/Kg bw	-	Anderson et al., 1980
HL*	NA	+	Pilinskaia, 1982
Micronuclei assay			
Cnesterodon decemmaculatus	50 – 157 mg/L	+	Vera Candioti et al., 2010b
Rhinella arenarum	80 – 250 mg/L	+	Vera Candioti et al., 2010a
Rat bone marrow cells	69.3 mg/Kg bw	-	Jones & Howard, 1989

Table 2. Evaluation of Pirimicarb-induced genotoxicity and cytotoxicity on different target systems. [a], expressed as reported by authors; UDS, unscheduled DNA synthesis; SCE, sister chromatid exchange; HL, human lymphocytes; CCP, cell-cycle proliferation; NA, data not available.

were respectively applied (Käfer et al., 1982; Callander, 1995). Negative and positive results were obtained for the induction of mutagenicity in mouse lymphoma L5178Y cells regardless of the presence or absence of a rat liver metabolic activation system (Clay, 1996). Furthermore, the induction of DNA single strand breaks, estimated by the alkaline comet assay, was evaluated revealing positive results in human lymphocytes exposed *in vitro* to pirimicarb (Ündeger & Basaran, 2005). Similar positive results were found when the sister chromatid exchange assay was performed in Chinese hamster ovary cells (Soloneski & Larramendy, 2010). Although a significant increase in chromosomal aberrations has been reported in Chinese hamster ovary cells after pirimicarb exposure (Soloneski & Larramendy, 2010), Wildgoose and coworkers (1987) observed negative results in human lymphocytes with or without S9 metabolic activation. Finally, the induction of alterations in the cell-cycle progression in Chinese hamster ovary cells was reported to occur after *in vitro* exposure to pirimicarb (Soloneski & Larramendy, 2010).

In *in vivo* genotoxic and cytotoxic studies, pirimicarb was able to induce different types of lesions. It has been reported the ability of the insecticide to give positive results by using the eye mosaic system *white/white*$^+$ (*w/w*+) somatic mutation and recombination test (SMART) when *Drosophila melanogaster* was employed as experimental model (Aguirrezabalaga et al., 1994). However, McGregor and co-workers (1974) reported negative results in mice when the dominant lethal mutation assay was performed. Similar negative results were found by Kenelly (1990) using the unscheduled DNA synthesis in rat liver cells. At the chromosomal level, pirimicarb did not induce chromosomal alterations in bone marrow cells of Wistar male rats after oral administration (Anderson et al., 1980). Contrarily, Pilinskaia (1982) observed a significant increase of chromosomal aberrations in the peripheral blood lymphocytes from occupational workers after pirimicarb exposure. Finally, when the micronuclei induction end-point was employed, positive results were reported in erythrocytes of the fish *Cnesterodon decemmaculatus* and *Rhinella arenarum* tadpoles by Vera Candioti and collaborators (Vera Candioti et al., 2010a, b). Lastly, when a mammal model was employed for the micronuclei detection, Jones and Howard (1989) found negative results in rat bone marrow cells.

5. Comparison of the genotoxicity and cytotoxicity of carbofuran and pirimicarb and some Argentinean technical formulations

One of the goals of our research group is to compare the genotoxic and cytotoxic effects exerted by the pesticide active ingredients (Pestanal®, Riedel-de Haën, Germany) and their technical formulations commonly used in Argentina on vertebrate cells both *in vitro* and *in vivo*.

The evaluation was performed using end-points for genotoxicity [Sister Chromatid Exchange, Chromosome Aberration, and Micronuclei frequencies] and cytotoxicity [Mitotic Index, Cell Viability, Proliferative Rate Index, Erythroblasts/Erythrocytes Ratio, 3(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Neutral Red assays] (Soloneski et al., 2008; Soloneski & Larramendy, 2010; Vera Candioti et al., 2010a, b).

We comparatively evaluated the genotoxic and cytotoxic *in vitro* effects on mammalian Chinese hamster ovary cells induced by the pure insecticide carbofuran and its commercial formulation Furadan® (47% carbofuran, FMC Argentina S.A., Buenos Aires, Argentina). Similarly, the genotoxic and cytotoxic *in vitro-in vivo* effects induced by the pure insecticide pirimicarb and its commercial formulation Aficida® (50% pirimicarb, Syngenta Agro S.A.,

Buenos Aires, Argentina) on mammalian Chinese hamster ovary cells as well as circulating erythrocytes of the fish *Cnesterodon decemmaculatus* and the amphibian *Rhinella arenarum* tadpoles were also estimated.

A summary of the results obtained is presented in Fig. 3. The figure clearly reveals that all compounds assayed were able to inflict damage at chromosomal and cellular level regardless of the cellular system used as target.

We observed that carbofuran/Furadan® and pirimicarb/Aficida® caused SCEs on mammalian cells indicating that they have a clastogenic activity (Fig. 3A). It has been suggested that at the chromosomal level, the induction of SCEs is a reliable indicator for the screening of clastogens, since the bioassay is more sensitive than the analysis of clastogen-induced chromosomal aberrations (Palitti et al., 1982). The results also demonstrate the ability of pirimicarb/Aficida® to induce DNA damage quali- and quantitative analyzed by the frequency of chromosomal aberrations (Fig. 3B). Furthermore, a putative clastogenic/aneugenic activity exerted *in vitro* by carbofuran/Furadan® and *in viv*o by pirimicarb/Aficida® was also demonstrated by the ability of the pesticide-induced micronuclei (Fig. 3C). The analysis of the proliferative replication (Fig. 3D) and the mitotic indexes (Fig. 3E) demonstrated that both carbofuran/Furadan® and pirimicarb/Aficida® were able to delay the cell-cycle progression as well as to exert a marked reduction of the cellular mitotic activity on mammalian cells *in vitro*. Besides, carbofuran/Furadan® and pirimicarb/Aficida® were able to induced a clear cellular cytotoxicity. This deleterious effect was estimated by a loss of lysosomal activity (indicated by a decrease in the uptake of neutral red), as well as alteration in energy metabolism (measured by mitochondrial succinic dehydrogenase activity in the MTT assay), as clearly revealed in insecticides-treated Chinese hamster ovary cells (Fig. 3F).

Overall, the results revealed, depending upon the endpoint employed, that the damage induced by the commercial formulations of both insecticides is, in general, greater than that produced by the pure pesticides (Fig. 3). Unfortunately, the identity of the components present in the excipient formulations was not made available by the manufacturers. These final remarks are in accord with previous observations not only reported by us but also by other research groups indicating the presence of xenobiotics within the composition of the commercial formulations with genotoxic and cytotoxic effects (David, 1982; Lin & Garry, 2000; Soloneski et al., 2001, 2002, 2003, 2008; González et al., 2007a, b, 2009; Elsik et al., 2008; Molinari et al., 2009; Soloneski & Larramendy, 2010). These observations highlight that, in agriculture, agrochemicals are generally not used as a single active ingredient but as part of a complex commercial formulations. Thus, both the workers as well as non-target organisms are exposed to the simultaneous action of the active ingredient and a variety of other chemical/s contained in the formulated product. Hence, risk assessment must also consider additional geno-cytotoxic effects caused by the excipient/s.

Finally, the results highlight that a whole knowledge of the toxic effect/s of the active ingredient of a pesticide is not enough in biomonitoring studies as well as that agrochemical/s toxic effect/s should be evaluated according to the commercial formulation available in market. Furthermore, the deleterious effect/s of the excipient/s present within the commercial formulation should be neither discarded nor underestimated. The importance of further studies on this type of pesticide in order to achieve a complete knowledge on its genetic toxicology seems to be, then, more than evident.

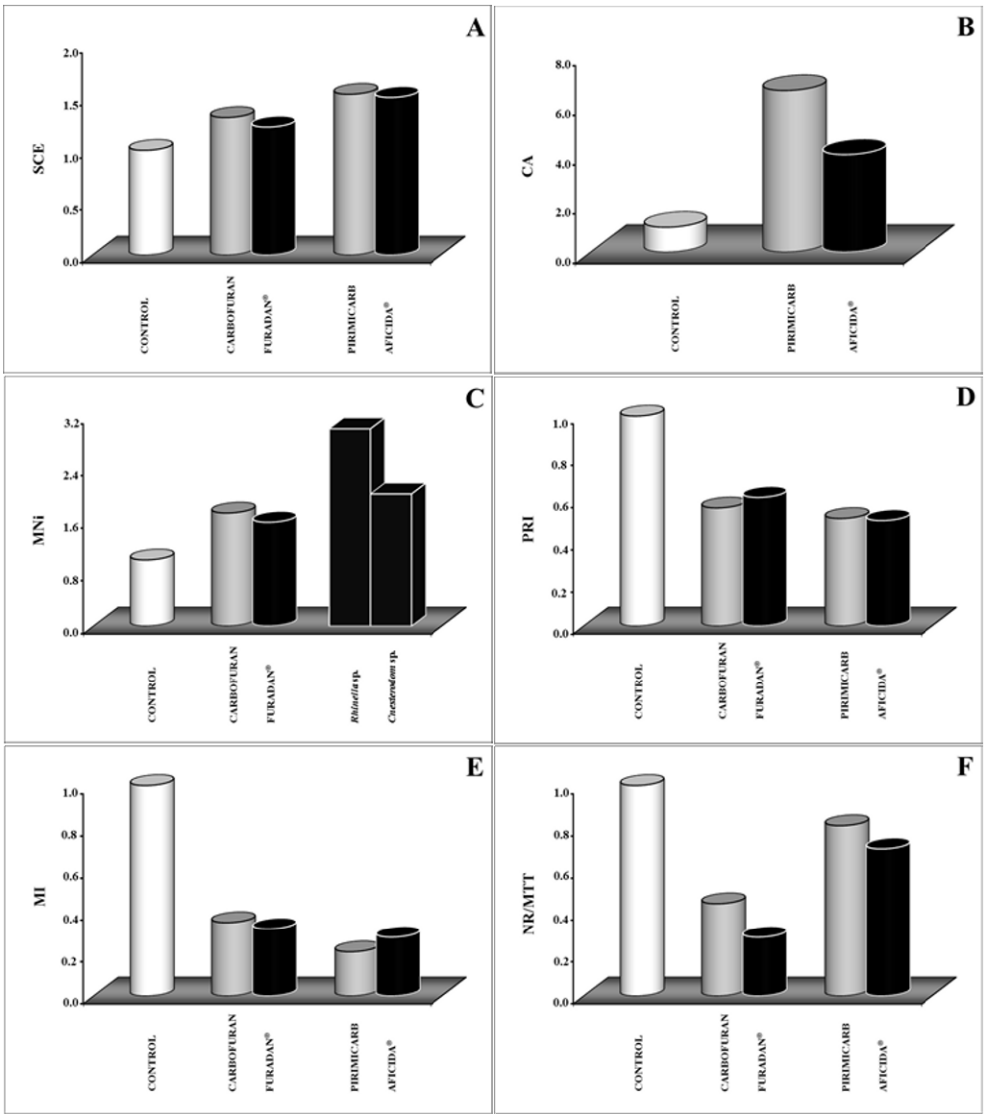

Fig. 3. Comparative genotoxicity and cytotoxicity effects induced by carbofuran and pirimicarb pure herbicides Pestanal® (grey) and their technical formulations Furadan® and Aficida® (black) commonly used in Argentina on mammalian Chinese hamster ovary cells (cylinders) and *in vivo* piscine and amphibian erythrocytes (bars). Results are expressed as fold-time values over control data. Evaluation was performed using end-points for genotoxicity [Sister Chromatid Exchanges (A), Chromosome Aberrations (B), Micronuclei (C)] and cytotoxicity [Proliferative Rate Index (D), Mitotic Index (E), 3(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Neutral Red (NR) (F)].

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7. References

Aguirrezabalaga, I., Santamaría, I., Comendador, M.A., 1994. The w/w+ SMART is a useful tool for the evaluation of pesticides. *Mutagenesis* 9, 341-346.

Aly, M.S., 1998. Chromosomal damage induced in adult mice by carbofuran. *Egyptian German Society of Zoology* 26C, 1-10.

Anderson, D., Richardson, C.R., Howard, C.A., Bradbrook, C., Salt, M.J., 1980. Pirimicarb: a cytogenetic study in the rat. World Health Organization.

Blevins, R.D., Lee, M., Regan, J.D., 1977a. Mutagenicity screening of five methyl carbamate insecticides and their nitroso derivatives using mutants of *Salmonella typhimurium* LT2. *Mutation Research* 56, 1-6.

Blevins, R.D., Lijinksy, W., Regan, J.D., 1977b. Nitrosated methylcarbamate insecticides: effect on the DNA of human cells. *Mutation Research* 44, 1-7.

Bolognesi, C., 2003. Genotoxicity of pesticides: a review of human biomonitoring studies. *Mutation Research* 543, 251–272.

Bolognesi, C., Carrasquilla, G., Volpi, S., Solomon, K.R., Marshall, E.J., 2009. Biomonitoring of genotoxic risk in agricultural workers from five colombian regions: association to occupational exposure to glyphosate. *Journal of Toxicological and Environmental Health. Part A* 72, 986-997.

Bolognesi, C., Creus, A., Ostrosky-Wegman, P., Marcos, R., 2011. Micronuclei and pesticide exposure. *Mutagenesis* 26, 19-26.

Bolognesi, C., Parrini, M., Reggiardo, G., Merlo, F., Bonassi, S., 1993. Biomonitoring of workers exposed to pesticides. *International Archives of Occupational and Environmental Health* 65, S185-S187.

Callander, R.D., 1995. Pirimicarb: an evaluation of the mutagenic potential using *S. typhimurium* and *E. coli*. Central Toxicology Laboratory Report No. CTL/P/4798 GLP, Unpublished.

Canna-Michaelidou, S., Nicolaou, A.S., 1996. Evaluation of the genotoxicity potential (by Mutatox test) of ten pesticides found as water pollutants in Cyprus. *The Science of the Total Environmental Research* 193, 27-35.

Cantelli-Forti, G., Paolini, M., Hrelia, P., 1993. Multiple end point procedure to evaluate risk from pesticides. *Environmental Health Perspectives* 101, 15-20.

Castillo-Cadena, J., Tenorio-Vieyra, L.E., Quintana-Carabia, A.I., García-Fabila, M.M., Ramírez-San Juan, E., Madrigal-Bujaidar, E., 2006. Determination of DNA damage in floriculturists exposed to mixtures of pesticides. *Journal of Biomedicine and Biotechnology* 2006, 1-12.

Clark, H.A., Snedeker, S.M., 2005. Critical evaluation of the cancer risk of dibromochloropropane (DBCP). *Journal of Environmental Science and Health - Part C Environmental Carcinogenesis and Ecotoxicology Reviews* 23, 215-260.

Clay, P., 1996. Pirimicarb: L5178Y TK+/- Mouse Lymphoma Mutation Assay. Central Toxicology Laboratory. Report No: CTL/P/5080 GLP, Unpublished.

Cole, D.C., Carpio, F., Julian, J., Léon, N., 1998. Assessment of peripheral nerve function in an Ecuadorian rural population exposed to pesticides. *Journal of Toxicology and Environmental Health. Part A* 55, 77-91.

Chauhan, L.K., Pant, N., Gupta, S.K., Srivastava, S.P., 2000. Induction of chromosome aberrations, micronucleus formation and sperm abnormalities in mouse following carbofuran exposure. *Mutation Research* 465, 123-129.

Das, A.C., Chakravarty, A., Sukul, P., Mukherjee, D., 2003. Influence and persistence of phorate and carbofuran insecticides on microorganisms in rice field. *Chemosphere* 53, 1033-1037.

David, D., 1982. Influence of technical and commercial decamethrin, a new synthetic pyrethroid, on the gonadic germ population in quail embryos. *Archives d'Anatomie, d'Histologie et d'Embryologie* Vol. 65, 99-110.

DeGraff, W.G., 1983. Mutagenicity evaluation of MC 10242 for the sex-linked recessive lethal test in *Drosophila melanogaster*. Study No. A 83-1060. Unpublished report prepared by Litton Bionetics, Inc. Kensington, MD, USA. Submitted to WHO by FMC Corp., Philadelphia, PA, USA.

Dorough, H.W., Casida, J.E., 1964. Insecticide metabolism, nature of certain carbamate metabolites of insecticide Sevin. *Journal of Agricultural and Food Chemistry* 12, 294-304.

Elsik, C.M., Stridde, H.M., Tann, R.S., 2008. Glyphosate adjuvant formulation with glycerin. ASTM Special Technical Publication, pp. 53-58.

EPA, 1974a. Compendium of Registered Pesticides. US Government Printing Office, Washington, DC.

EPA, 1974b. Pesticide Fact Sheet: Pirimicarb. US Government Printing Office, Washington, DC.

EPA, 2004. ECOTOX: Ecotoxicology Database. USEPA/ORD/NHEERL, Mid-Continent Ecology Division. URL:http://www.epa.gov/ecotox/ecotox_home.htm.

EPA, 2006. Carbofuran I.R.E.D. Facts. U.S. Environmental Protection Agency, Office of Pesticide Programs, U.S. Government Printing Office: Washington, DC.

EPA, 2007. Revised *N*-methyl carbamate cumulative risk assessment. U.S. Environmental Protection Agency. Office of Pesticide Programs.

FMC, 1971. Dominant-lethal mutagenicity study [carbofuran]. NCT. 438.99. DPR Vol. 254-029, Record 939879.

Gee, J., 1983. *Drosophila* sex-linked recessive lethal assay of 2,3dihydro-2,2-dimethyl-7-benzofuranyI N-methyl carbamate (carbofuran, T-2047). University of Wisconsin. Lab. Project 103c. DPR Vol. 254-109, Record 47742.

Gentile, J.M., Gentile, G.J., Bultman, J., Sechriest, R., Wagner, E.D., Plewa, M.J., 1982. An evaluation of the genotoxic properties of insecticides following plant and animal activation. *Mutation Research* 101, 19-29.

Georgian, L.I., Moraru, I., Draghicescu, T., Tarnavschi, R., 1985. The effect of low concentrations of carbofuran, cholin salt of maleic hydrazine, propham and

chlorpropham on sister-chromatid exchange (SCE) frequency in human lymphocytes *in vitro*. *Mutation Research* 147, 296-301.

González, N.V., Soloneski, S., Larramendy, M., 2009. Dicamba-induced genotoxicity oh Chinese hamster ovary (CHO) cells is prevented by vitamin E. *Journal of Hazardous Materials* 163, 337-343.

González, N.V., Soloneski, S., Larramendy, M.L., 2007a. The chlorophenoxy herbicide dicamba and its commercial formulation banvel induce genotoxicity in Chinese hamster ovary cells. *Mutation Research* 634, 60-68.

González, N.V., Soloneski, S., Larramendy, M.L., 2007b. Genotoxicity analysis of the phenoxy herbicide dicamba in mammalian cells *in vitro*. *Toxicology In Vitro* 20, 1481-1487.

Gupta, R.C., 1994. Carbofuran toxicity. *Journal of Toxicology and Environmental Health. Part A* 43, 383-418.

Haworth, S.R., Lawlor, T.E., 1983. *Salmonella*/mammalian microsome plate incorporation mutagenicity assay (Ames test). Carbofuran technical. FMC Study No. A 83-868 (MBA Study No. 1921.501). Unpublished report prepared by Microbiological Associates, MD, USA.Submitted to WHO by FMC Corp., Philadelphia, PA, USA.

Hodgson, E., Levi, P.E., 1996. Pesticides: an important but underused model for the environmental health sciences. *Environmental Health Perspectives* 104, 97-106.

Hour, T.C., Chen, L., Lin, J.K., 1998. Comparative investigation on the mutagenicities of organophosphate, phthalimide, pyrethroid and carbamate insecticides by the Ames and lactam tests. *Mutagenesis* 13, 157-166.

HSDB, 2011. Carbofuran. National Library of Medicine.

IARC, 1976. Some carbamates, thiocarbamates and carbazides. International Agency for Research on Cancer, Lyon.

IARC, 1987. Genetic and related effects: an updating of selected IARC monographs from volumes 1 to 42. International Agency for Research on Cancer, Lyon.

IARC, 2003. Monographs on the evaluation of carcinogenic risk to human. Vols. 5 - 53. International Agency for Research on Cancer, Lyon.

Jones, K., Howard, C.A., 1989. Pirimicarb (technical): an evaluation in the mouse micronucleus test. Unpublished report No. CTL/P/2641 from Central Toxicology Laboratory, Zeneca. Submitted to WHO by Syngenta Crop Protection AG. Conducte according to OECD 474 (1983). GLP compliant.

Jung, Y.S., Kim, C.S., Park, H.S., Sohn, S., Lee, B.H., Moon, C.K., Lee, S.H., Baik, E.J., Moon, C.H., 2003. N-nitroso carbofuran induces apoptosis in mouse brain microvascular endothelial cells (bEnd.3). *Journal of Pharmacology Sciences* 93, 489-495.

Käfer, E., Scott, B.R., Dorn, G.L., Stafford, R., 1982. *Aspergillus nidulans*: systems and results of tests for chemical induction of mitotic segregation and mutation. I. Diploid and duplication assay systems. A report of the U.S. EPA Gene-Tox Program. *Mutation Research* 98, 1-48.

Kamel, F., Boyes, W.K., Gladen, B.C., Rowland, A.S., Alavanja, M.C., Blair, A., Sandler, D.P., 2000. Retinal degeneration in licensed pesticide applicators. *American Journal of Industrial Medicine* 37, 618-628.

Karczmar, A., 1998. Anticholinesterases: dramatic aspects of their use and misuse. *Neurochemistry International* 32, 401-411.

Kennelly, J.C., 1990. Pirimicarb: Assessment for the induction of unscheduled DNA synthesis in rat hepatocytes *in vivo*. Unpublished report No. CTL/P/2824 from Central Toxicology Laboratory, Zeneca. Submitted to WHO by Syngenta Crop Protection AG. Conducte according to OECD 486 (1983). GLP compliant.

Kim, S.J., Kim, J.E., Ko, B.H., Moon, I.S., 2004. Carbofuran induces apoptosis of rat cortical neurons and down-regulates surface alpha7 subunit of acetylcholine receptors. *Molecules and Cells* 17, 242-247.

Kirby, P.E., 1983a. L5178Y TK+/- mouse lymphoma mutagenesis assay. FMC Study No. A 83-962 and A 83-988 (MBA Study No. T1982.701). Unpublished report prepared by Microbiological Associates, Bethesda, MD, USA. Submitted to WHO by FMC Corp., Philadelphia, PA, USA.

Kirby, P.E., 1983b. L5178Y TK+/- mouse lymphoma mutagenesis assay. FMC Study No. A 83-1064 (MBA Study No. T2124.201). Unpublished report prepared by Microbiological Associates, Bethesda, MD, USA. Submitted to WHO by FMC Corp., Philadelphia, PA, USA.

Lin, C.M., Wei, L.Y., Wang, T.C., 2007. The delayed genotoxic effect of *N*-nitroso *N*-propoxur insecticide in mammalian cells. *Food and Chemical Toxicology* 45, 928-934.

Lin, N., Garry, V.F., 2000. *In vitro* studies of cellular and molecular developmental toxicity of adjuvants, herbicides, and fungicides commonly used in Red River Valley, Minnesota. *Journal of Toxicology and Environmental Health* 60, 423-439.

McGregor, D.B., 1974. Dominant lethal study in mice of ICI PP062. Zeneca unpublished report No. CTL/C/256 from Inveresk Research International. Submitted to WHO by Syngenta Crop Protection AG. Conducte according to OECD 478 (1983). GLP compliant.

Metcalf, R., Fukuto, R., Collins, C., Borck, K., Abd El-Aziz, A., Munoz, R., Cassil, C., 1968. Metabolism of 2,2-dimethyl-2,3-dihydrobenzofuran-7-N-methylcarbamate (Furadan) in plants, insects, and mamals. *Journal of Agricultural and Food Chemistry* 16, 300-311.

Mladinic, M., Perkovic, P., Zeljezic, D., 2009. Characterization of chromatin instabilities induced by glyphosate, terbuthylazine and carbofuran using cytome FISH assay. *Toxicology Letters* 189, 130-137.

Molinari, G., Soloneski, S., Reigosa, M.A., Larramendy, M.L., 2009. *In vitro* genotoxic and citotoxic effects of ivermectin and its formulation ivomec® on Chinese hamster ovary (CHO_{K1}) cells. *Journal of Hazardous Materials* 165, 1074-1082.

Moriya, M., Ohta, T., Watanabe, K., Miyazawa, T., Kato, K., Shirasu, Y., 1983. Further mutagenicity studies on pesticides in bacterial reversion assay systems. *Mutation Research* 116, 185-216.

Naravaneni, R., Jamil, K., 2005. Cytogenetic biomarkers of carbofuran toxicity utilizing human lymphocyte cultures *in vitro*. *Drug and Chemical Toxicology* 28, 359-372.

Palitti, F., Tanzarella, C., Cozzi, R., Ricordy, R., Vitagliano, E., Fiore, M., 1982. Comparison of the frequencies of SCEs induced by chemical mutagens in bone-marrow, spleen and spermatogonial cells of mice. *Mutation Research* 103, 191-105.

Pavanello, S., Clonfero, E., 2000. Biomarkers of genotoxic risk and metabolic polymorphisms. *Indicatori biologici di rischio genotossico e polimorfismi metabolici* 91, 431-469.

Peter, J.V., Cherian, A.M., 2000. Organic insecticides. *Anaesthesia and Intensive Care* 28, 11-21.

Pilinskaia, M.A., 1982. Cytogenetic effect of the pesticide pirimor in a lymphocyte culture of human peripheral blood *in vivo* and *in vitro*. *Tsitologiia Genetika* 16, 38-42.

Pilinskaia, M.A., Stepanova, L.S., 1984. Effect of the biotransformation of the insecticide furadan on *in vivo* and *in vitro* manifestations of its cytogenetic activity. *Tsitologiia Genetika* 18, 17-20.

Putman, D.L., 1983a. Activity of FMC 10242 (T1982) in the *in vivo* cytogenetics assay in sprague-dawley rats. Unpublished report No: A83-972 prepared by Microbiological Associates, Bethesda, MD, USA. Submitted to WHO by FMC Corp., Philadelphia, PA, USA.

Putman, D.L., 1983b. Activity of FMC 10242 in the subchronic *in vivo* cytogenetics assay in male rats. Study No. A 83-1065 (MBA No. T2124.102). Unpublished report prepared by Microbiological Associates, Bethesda, MD, USA. Submitted to WHO by FMC Corp., Philadelphia, PA, USA USA.

Saxena, P.N., Gupta, S.K., Murthy, R.C., 2010. Carbofuran induced cytogenetic effects in root meristem cells of *Allium cepa* and *Allium sativum*: A spectroscopic approach for chromosome damage. *Pesticide Biochemistry and Physiology* 96, 93-100.

Saxena, S., Ashok, B.T., Musarrat, J., 1997. Mutagenic and genotoxic activities of four pesticides: captan, foltaf, phosphamidon and furadan. *Biochemistry and Molecular Biology International* 41, 1125-1136.

Shirasu, Y., 1975. Significance of mutagenicity testing on pesticides. *Environmental and Quality Safety* 4, 226-231.

Simmon, U.F., 1979. *In vitro* microbiological mutagenicity and unscheduled DNA synthesis studies of eighteen pesticides. Study No. 68-01-2458 (FMC Study No. A81-509, EPA 600/1-79-041). Report prepared by SRI International, Menlo Park, California, for Health Effects Research Laboratory Office of Research and Development. US Environmental Protection Agency, Research Triangle Park, NC, USA. Submitted to WHO by FMC Corp., Philadelphia, PA.

Soloneski, S., A, R.M., Larramendy, M.L., 2003. Effect of the dithiocarbamate pesticide zineb and its commercial formulation, the azzurro. V. Abnormalities induced in the spindle apparatus of transformed and non-transformed mammalian cell lines. *Mutation Research* 536, 121-129.

Soloneski, S., González, M., Piaggio, E., Apezteguía, M., Reigosa, M.A., Larramendy, M.L., 2001. Effect of dithiocarbamate pesticide zineb and its commercial formulation

azzurro. I. Genotoxic evaluation on cultured human lymphocytes exposed *in vitro*. *Mutagenesis* 16, 487-493.

Soloneski, S., González, M., Piaggio, E., Reigosa, M.A., Larramendy, M.L., 2002. Effect of dithiocarbamate pesticide zineb and its commercial formulation azzurro. III. Genotoxic evaluation on Chinese hamster ovary (CHO) cells. *Mutation Research* 514, 201-212.

Soloneski, S., Larramendy, M.L., 2010. Sister chromatid exchanges and chromosomal aberrations in chinese hamster ovary (CHO-K1) cells treated with the insecticide pirimicarb. *Journal of Hazardous Materials* 174, 410-415.

Soloneski, S., Reigosa, M.A., Molinari, G., González, N.V., Larramendy, M.L., 2008. Genotoxic and cytotoxic effects of carbofuran and furadan® on Chinese hamster ovary (CHO_{K1}) cells. *Mutation Research* 656, 68-73.

SRI, 1979. *In vitro* microbiological mutagenicity and unscheduled DNA synthesis studies of eighteen pesticides. Contract 68-0102458. DPR Vol. 254-110, Record 47751 *(E. coli* & *B. subtilis),* 47752 *(S. cerevisiae),* 47753 (WI-38).

Stehrer-Schmid, P., Wolf, H., 1995. Effects of benzofuran and seven benzofuran derivatives including four carbamate insecticides in the *in vitro* porcine brain tubulin assembly assay and description of a new approach for the evaluation of the test data. *Mutation Research/Reviews in Genetic Toxicology* 339, 61-72.

Thilagar, A., 1983a. Chromosome aberrations in Chinese hamster ovary (CHO) cells. FMC Study No. A 83-1096. Unpublished report prepared by Microbiological Associates, Bethesda, MD, USA. Submitted to WHO by FMC Corp., Philadelphia, PA, USA.

Thilagar, A., 1983b. Sister chromatid exchange assay in Chinese hamster ovary (CHO) cells. FMC Study No. A 83-1095 (MBA No.1982.334001). Unpublished report prepared by Microbiological Associates, Bethesda, MD, USA. Submitted to WHO by FMC Corp., Philadelphia, PA, USA.

Thilagar, A., 1983c. Sister chromatid exchange assay in Chinese hamster ovary (CHO) cells. FMC Study No. A 83-1097 (MBA No. 2124.334001). Unpublished report prepared by Microbiological Associates, Bethesda, MD, USA. Submitted to WHO by FMC Corp., Philadelphia, PA, USA.

Thilagar, A., 1983d. Unscheduled DNA synthesis in rat primary hepatocytes. FMC Study No. A 83-969 (MBA No. T1982.380) Unpublished report prepared by Microbiological Associates, Bethesda, MD, USA. Submitted to WHO by FMC Corp., Philadelphia, PA, USA.

Trueman, R.W., 1980. An examination of pirimicarb for potential mutagenicity using the *Salmonella*/microsome reverse mutation assay. Unpublished report No: CTL/P/540 form Central Toxicology Laboratory, Zeneca. Submitted to WHO by Syngenta Crop Protection AG. Conducted to a protocol that was consistent with the OECD guideline 471 (1983). GLP compliant.

Ündeger, Ü., Basaran, N., 2005. Effects of pesticides on human peripheral lymphocytes *in vitro*: induction of DNA damage. *Archives in Toxicology* 79, 169-176.

Valencia, R., 1981. Mutagenesis screening of pesticides using *Drosophila*. FMC Study No. A 83-1042 (EPA 600/1-81-017). Report prepared by Warf Institute Inc., Madison, WI,

for Health Effects Research Laboratory. Office of Research and Development, US Environmental Protection Agency, Research Triangle Park, NC, USA. Submitted to WHO by FMC Corp., Philadelphia, PA, USA.

Valencia, R., 1983. *Drosophila* sex-linked recessive lethal assay of 2,3-dihydro-2,2-dimethyl-7-benzofuranyl- *N*-methyl carbamate (carbofuran). FMC Study No. A 83-1019 (MBA No. T1047.160). Unpublished report prepared by Microbiological Associates, Bethesda, MD and the University of Wisconsin, Zoology Department, USA. Submitted to WHO by FMC Corp., Philadelphia, PA, USA.

Vera Candioti, J., Natale, G.S., Soloneski, S., Ronco, A.E., Larramendy, M.L., 2010a. Sublethal and lethal effects on *Rhinella arenarum* (Anura, Bufonidae) tadpoles exerted by the pirimicarb-containing technical formulation insecticide Aficida. *Chemosphere* 78, 249-255.

Vera Candioti, J., Soloneski, S., Larramendy, M.L., 2010b. Genotoxic and cytotoxic effects of the formulated insecticide aficida on *Cnesterodon decemmaculatus* (Jenyns, 1842) (Pisces: Poeciliidae). *Mutation Research* 703, 180-186.

Waters, M.D., Sandhu, S.S., Simon, V.F., 1982. Study of pesticide genotoxicity. *Basic Life Sciences* 21, 275-326.

WHO-FAO, 2004. Pesticides residues in food-2004. FAO Plant Production and Protection paper World Health Organization and Food and Agriculture Organization of the United Nations, Rome, pp. 154-161.

WHO-FAO, 2009. Pesticides residues in food-2009. FAO Plant Production and Protection paper World Health Organization and Food and Agriculture Organization of the United Nations, Rome, pp. 1-426.

WHO, 1988. The WHO recommended classification of pesticides by hazard and guidelines to the classification 1988-1989. World Health Organization, Geneva.

WHO, 1990. Public health impacts of pesticides used in agriculture (WHO in collaboration with the United Nations Environment Programme, Geneva, 1990). World Health Organization, Geneva.

WHO, 2000-2002. International programme of chemical safety. World Health Organization, Geneva.

WHO, 2009. The WHO recommended classification of pesticides by hazard. World Health Organization, Geneva.

Wildgoose, J., Howard, C.A., Richardson, C.R., Randall, V., 1987. Pirimicarb: A cytogenetic study in human lymphocytes *in vitro*. Central Toxicology Laboratory. Report No. CTL/P/1655, GLP, Unpublished.

Wojciechowski, J.P., Kaur, P., Sabharwal, P.S., 1982. Induction of ouabain resistance in V-79 cells by four carbamate pesticides. *Environmental Research* 29, 48-53.

Woodruff, R.C., Phillips, J.P., Irwin, D., 1983. Pesticide-induced complete and partial chromosome loss in screens with repair-defective females of *Drosophila melanogaster*. *Environmental Mutagenesis* 5, 835-846.

Yoon, J.Y., Oh, S.H., Yoo, S.M., Lee, S.J., Lee, H.S., Choi, S.J., Moon, C.K., Lee, B.H., 2001. *N*-nitrosocarbofuran, but not carbofuran, induces apoptosis and cell cycle arrest in CHL cells. *Toxicology* 169, 153-161.

Zeljezic, D., Vrdoljak, A.L., Lucas, J.N., Lasan, R., Fucic, A., Kopjar, N., Katic, J., Mladinic, M., Radic, B., 2009. Effect of occupational exposure to multiple pesticides on translocation yield and chromosomal aberrations in lymphocytes of plant workers. *Environmental Sciences Technololgy* 43, 6370-6377.

Zhou, P., Liu, B., Lu, Y., 2005. DNA damaging effects of carbofuran and its main metabolites on mice by micronucleus test and single cell gel electrophoresis. *Science in China. Series C, Life Sciences* 48, 40-47.