TESTING VAPOR TOXICITY OF FORMATE, ACETATE, AND HETEROBICYCLIC COMPOUNDS TO *Aedes aegypti* AND *Musca domestica*

By

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Volatile insecticides, commonly known as fumigants, have been widely used for the management of structural pests and for the protection of stored agricultural commodities. However, they have been mostly overlooked for the control of medically important pests such as mosquitoes and flies. Dichlorvos (DDVP) is the one volatile insecticide mostly studied on mosquitoes and flies. DDVP has been characterized by the Environmental Protection Agency of the United States as a "probable human carcinogen" and because of its implications in human health, in 2006 its use was restricted to confined spaces such as wardrobes and closets. Therefore, there is need to replace DDVP with friendlier and less toxic chemistries.

For my research I evaluated vapor toxicity of a series of new, promising, highly volatile chemicals with insecticidal activity, low mammalian toxicity, pleasant odors, and potentially novel modes of action on mosquitoes, using *Aedes aegypti* (L.), and on filth flies, using *Musca domestica* (L.). A total of 16 insecticidal compounds, 7 formates, 4 acetates, 4 heterobicyclics, and the organophosphate DDVP were tested on mosquitoes. DDVP was by far the most toxic compound, and specifically it was 54.4 times more toxic than the second best performing compound, the formate ester methyl formate. Within the novel compounds, overall, formate esters were the most toxic family, followed by the heterobicyclics, and last by the acetate esters.

The seven best performing novel compounds with vapor toxicity on mosquitoes were methyl formate>butyl formate>propyl formate=ethyl formate>hexyl formate>coumaran>benzothiophene. There were several structure-activity relationships observed. The most striking one involved the length of the aliphatic chain of the formate esters; as the length of the aliphatic chain increased, toxicity in general decreased. Also, the formate group within the aliphatic chain was correlated with higher toxicity than the acetate group.

A total of 4 compounds, the formate esters heptyl formate and ethylene glycol di-formate (EGDF), the heterobicyclic menthofuran, and the organophosphate DDVP were tested on house flies. DDVP was 25 times more toxic compared to the second best compound, the heterobicyclic menthofuran. Menthofuran was followed by EGDF, and last by heptyl formate. Also, ceramic porous rods were embedded with heptyl formate in order to evaluate the effectiveness of controlled vapor release of heptyl formate in killing house flies over time. It was shown that controlled vapor release of heptyl formate can be used successfully to provide house fly mortality over time.

Three of the novel compounds, heptyl formate, EGDF, and menthofuran were synergized with the insecticide synergists SSS-tributyl-phosphorotrithioate (DEF), and piperonyl butoxide (PBO), which are esterase and P450 inhibitors, respectively. For both mosquitoes and house flies, when EGDF and heptyl formate were co-applied with DEF their toxicities decreased, supporting esterase based activation of formate esters. Also, when menthofuran was synergized with PBO its toxicity increased, supporting P450 based deactivation of heterobicyclics.

CHAPTER 1
INTRODUCTION

It would be impossible to refer to all those times that insects affected the course of human history. Just to name a few, the death of great warriors like Alexander the Great was attributed to malaria, a disease transmitted by mosquitoes. Great empires like the Roman Empire were brought to decline because of the Bubonic plague, a disease transmitted by fleas. Of course not to forget that Bubonic plague, also referred to as the Black Death, was responsible for causing 25 to 75 million deaths in Europe alone. A vast number of deaths during wars is attributed to insect borne diseases; in the American civil war an estimate of 40,000 to 100,000 deaths were attributed to dysentery, a disease transmitted by filth-flies (Capinera 2004).

Because of the major impact insects can have on people's lives, people have had an ongoing battle with them since the very early years in the pages of history. The first human attempt to control insects was documented during the early years of ancient Greece. Homer described how Odysseus fumigated a house with burning sulfur to control insect pests (Homer, 800 B.C.E). Since then, there has been a lot of improvement in the development of chemical compounds with effective insecticidal activities. The first successful compound with phenomenal insecticidal activity was the chlorinated hydrocarbon, DDT (dichlorodiphenyltrichloroethane) (Casida & Quistad 1998). DDT was brought into the insecticide market in 1939, and it was effective against a wide range of insects and most notably against mosquitoes. Paul Muller received a Nobel Prize in 1949 for discovering the insecticidal activities of DDT (Capinera 2004). After the development of the chlorinated hydrocarbons other successful insecticidal groups followed, such as the organophosphates [parathion (1946), malathion (1952), chlorpyrifos (1965)], the methyl carbamates [carbaryl (1957), alanycarb (1984)], and the pyrethroids [allethrin (1949), resmethrin (1967), permethrin (1973), deltamethrin (1974)] (Casida & Quistad

1998). Some more recent insecticidal groups are the insect growth regulators such as juvenoids and chitin synthesis inhibitors [methoprene (1973), fenoxycarb (1981),] the chloronicotinyls [imidacloprid (1990)], the phenylpyrazoles [fipronil (1992)], and the avermectins [abamectin (1981)] (Casida & Quistad 1998).

All these insecticides have had a wide application spectrum targeting various insect pests, including agricultural and public health pests. My research will specifically focus on two of the most important categories of public health pests: the mosquitoes (Diptera: Culicidae) and the filth flies (Diptera: Muscidae). Each one of these pests has its own life history, unique behavioral and morphological traits, and different potential for disease transmission. Mosquitoes, despite their miniature size, and their delicate, vulnerable figure have managed successfully to survive on planet earth for more than 170 million years. With their unique adaptation mechanisms they have managed to thrive in almost all kinds of water habitats, from crab-holes and leaf-axils, to subzero tundra wetlands in Arctic. Mosquitoes are vectors of serious and deadly diseases such as malaria, yellow fever, dengue and the different types of encephalitis. A total number of approximately 320 million human cases of mosquito borne diseases with 2 million deaths occur every year (Tabachnick 2004). There are approximately 3,200 recognized mosquito species worldwide and the largest number of them still remains to be discovered (Rutledge 2004). The mosquito species that was studied in this research was the yellow fever mosquito *Aedes aegypti* (L). Like all dipterans, mosquitoes exhibit holometabolous development. Their life cycle is completed in two different environments: one aquatic and one terrestrial. The first three stages of their life cycle, the egg, the larva, and the pupa, are adapted to survive in aquatic environments, whereas the last stage, the winged adult inhabits terrestrial environments. Their life-cycle lasts from 7 to 14 days depending on the mosquito species. A more detailed description of the species'

morphology, behavior, biology and a review of the current control methods will be given in Chapter 2.

Filth flies have been in close association with humans since humans showed up on planet Earth. They have been a nuisance with their painful bites and a plague due to the serious, life threatening diseases they transmit. Some of the diseases they transmit are typhoid fever, dysentery and diarrhea. Flies are also known to infect human and animal flesh, a condition known as myiasis. There are approximately 87,000 species of flies worldwide (not including mosquitoes) (Scott & Littig 1964) and 9,000 of them belong to the family Muscidae (Mullen & Durden 2002). The filth fly species that was studied in this research was the common house fly *Musca domsestica* (L.). Houseflies have complete metamorphosis, and their life cycle is divided into 4 stages: the egg, the larva, the pupa, and the winged adult. The time necessary for the completion of the cycle depends on the species and on the environmental conditions such as temperature and moisture. A more detailed description of the species' morphology, behavior, biology, and a review of current control methods will be given in Chapter 3.

A major problem that emerged from the use of insecticides is the development of resistance. It was Melander (1914) that first reported insecticide resistance. Since then the number of insects and mites worldwide that have developed resistance to one or more pesticides has increased to 504 and continues to increase (Becker 2003). Specifically, the number of public health pests that developed insecticide resistance has increased from 2 in 1946, to 198 in 1990 (Oppenoorth 1985, Georghiou 1990). Both mosquitoes and houseflies developed resistance rapidly to various insecticides. Hemingway and Ranson (2000) gave a very nice review of insecticide resistance on mosquitoes that vector diseases. In 1947 the first case of DDT resistance was documented in *Aedes tritaeniorhynchus* and *Aedes solicitans*. Since then more

than 100 mosquito species have developed resistance to one or more of the insecticides discussed above. A broad-spectrum of organophosphate resistance or malathion-specific resistance has been documented in the major malaria vectors (*Anopheles* group) such as *Anopheles culicifacies, Anopheles stephensi, Anopheles albimanus, Anopheles arabiensis, Anopheles sacharovi.* Also, pyrethroid resistance has occurred in *Anopheles albimanus, Anopheles stephensi*, and *Anopheles gambiae* among others, not to neglect the carbamate resistance in *Anopheles sacharovi* and *Anopheles albimanus*. Widespread resistance to organophosphates has occurred in the *Culex* group as well, and pyrethroid resistance was recorded in *Culex quienquefasciatus*. Widespread resistance to pyrethroids has occurred in *Aedes aegypti*, and additionally many cases of carbamate and organophosphate resistance have been recorded as well. Things do not look any better for house flies. It was again in the year of 1947 that the first case of house fly resistance to DDT was recorded (Georghiou 1972). Keiding 1999 prepared a very nice review of the global status of insecticide resistance in field populations of the housefly, *Musca domestica* (L.). According to his review, when the organochlorines failed to control flies (1950), they were replaced by the organophosphorous compouds, and it wasn't long afterwards that organophosphorous resistance was recorded (1955, Denmark). It didn't take long to spread to different parts of the world (North America 1966, United Kingdom 1977, Germany 1979, Japan 1979, Belgium 1981, West Africa 1979, Australia 1989 to name a few). Widespread resistance to carbamates was also seen, with an early Czechoslovakian report in 1983. Resistance on pyrethroids was first recorded in Denmark in the 1970's. It is, also, worth mentioning that in the USA, the first pyrethroid resistance case was observed in 1984 in Georgia after only 2 years of permethrin use.

Considering the limited number of insecticides registered for management of public health insect pests and the increasing incidents of resistance documented, it is essential that already existing insecticides must be used wisely and that new insecticides with novel modes of action must be discovered. The main objective of this study is to evaluate a series of new promising chemicals with insecticidal activity and potentially novel modes of action on mosquitoes, using *Aedes aegypti* (L.), and on filth flies, using *Musca domestica* (L.). These new insecticides have high vapor pressures, and, as a result they show potential to act as vapor toxicants. The experiments presented in this paper evaluated vapor toxicity of the novel insecticidal chemistries.

CHAPTER 2
LITERATURE REVIEW: THE YELLOW FEVER MOSQUITO

> No animal on earth has touched so directly and profoundly the lives of so many human beings. For all of history, and all over the globe, she has been a nuisance, a pain, and an angel of death. The mosquito has killed great leaders, decimated armies, and decided the fate of nations. All this, and she is roughly the size and weight of a grape seed. (Spielman and D'Antonio 2001, p. 15 from The Preface to Mosquito)

Classification and Distribution

Aedes aegytpi (L.) is a mosquito species in the family Culicidae, subfamily Culicinae, and tribe Aedini. There are three types of this species: the typical form *Ae. aegypti aegytpi*, *Ae. aegytpi queenslandensis*, and the smallest type *Ae. aegytpi formosus* which is a forest species (Nelson 1986). Only the first two types are found in the USA.

World distribution. *Ae. aegypti* is thought to have originated from Africa (Gratz 1993). It has been introduced to many parts of the world through ships and therefore ports are the first areas to be invaded. Currently this species is distributed in most tropical and subtropical world regions, with a range extending from 40 degrees N to 40 degrees S latitude (Womack 1993).

USA distribution. *Ae. aegypti* occurs in 21 states, which are Alabama, Mississippi, Florida, Georgia, Tennessee, Kentucky, North Carolina, South Carolina, Virginia, New York, Delaware, Maryland, Kansas, District of Columbia, Illinois, Arkansas, Louisiana, Missouri, Oklahoma, Texas, and New Mexico (Womack 1993, Darsie and Ward 2005).

Florida distribution. *Ae. aegypti* used to be widely distributed through the entire state of Florida (Tinker and Hayes 1959, Morlan and Tinker 1965). However, since the introduction of *Ae. albopictus* in 1986 (Peacock et al. 1988), a significant decline of the *Ae. aegytpi* population has been detected (O'Meara et al. 1992a, O'Meara et al. 1995).

Morphology

The Egg

The eggs are black in color, cigar shaped and one millimeter in length.

The Larva

The body of the larva is divided into 3 distinct segments; the head, the thorax and the abdomen. The head and thorax have an ovoid shape and the abdomen is divided into nine segments. The posterior segment of the abdomen has four specially modified gills for osmotic regulation and a siphon specialized for breathing (Mullen and Durden 2002). The siphon of the *Aedes* mosquitoes is distinctively shorter than other mosquitoes and plays an important role in distinguishing them from others. Also, the position of the *Aedes* larvae in water is almost vertical to the water surface (Nelson 1986).

Two distinctive characteristics set *Ae. aegytpi* apart from other *Aedes* larvae. The first one is the two prominent lateral hooks (spines) on each side of the thorax. The second one is the row of seven to twelve comb scales on the eighth abdominal segment. Each one of these scales has two lateral teeth and a medial spine that gives it a 'pitchfork' appearance (Nelson 1986, Darsie and Ward 2005).

The Pupa

Pupae in the genus of *Aedes* have a distinct short hair at the tip of each swimming paddle and short breathing tubes known as air trumpets (Nelson 1986).

The Adult

Aedes aegypti is a medium sized black colored mosquito with a distinctive lyre-shaped design on the mesonotum. It also has white bands at the bases of the tarsal segments. Another key characteristic is the white segments on the palpi and the clypeus (Christophers 1960, Darsie and Ward 2005).

Life Cycle

The Egg

Aedes aegypti were originally tree-hole breeders (Soper 1967), but as they evolved and adapted in environments near and around human dwellings they became container breeders. A unique characteristic of their biology is that they attach their eggs on the sides of artificial as well as natural containers (Pratt and Littig 1967, Nelson 1986, Mullen and Darden 2002, Becker 2003, Rutledge and Evans. 2004). The eggs are fertilized at the moment of oviposition and it takes from 48 hours up to 5 days for embryonic development to be completed depending on the environmental temperature (Nelson 1986). The eggs have the ability to withstand long desiccation periods for up to one year and sometimes even more. Temperature and humidity play a significant role in the viability of the eggs. It was shown that at relative humidities from 91% to 95% *Ae. aegypti* embryos can survive for up to 15th months (Christophers 1960). Also, temperatures ranging from 42^{o} to 53^{o} F were shown to be lethal to the embryo when the eggs were exposed to them for more than 2 weeks. Flooding is the necessary stimulus for the eggs to hatch. It takes 15 minutes of flooding for some eggs to hatch. On the other hand some eggs need to be inundated several times prior to hatching (Nelson 1986).

The Larva

The larval development is divided into 4 instars. The first three instars develop fast and are more sensitive whereas the last instar takes longer to develop and increases more in size and weight (Nelson 1986, Mullen and Durden 2002). The duration of the development depends on several factors such as food availability, environmental temperature and larval density (Cristophers 1960, Gerberg et al. 1994). It can vary from as short as 5 days at optimal conditions up to 14 days. At a constant temperature of 21-25^{o} C the larvae are expected to pupate at 10-12 days (Geberg et al. 1994). Under unfavorable conditions the duration of the last instar can last for

up to several weeks before pupation takes place (Nelson 1986). The male larvae develop faster than the female larvae, and as a result they pupate one day earlier (Mullen and Durden 2002).

The Pupa

The pupae are the least active stage. They do not feed and their main function is metamorphosis into the mature adult stage (Nelson 1986, Mullen and Durden 2002). The pupae have the ability to react to external stimuli such as vibrations and light, and thus actively move away. The pupae are buoyant, and therefore they have the ability to float on the water. This property allows them to emerge as adults. The duration of the pupal stage lasts 2 to 3 days (Nelson 1986, Mullen and Durden 2002). The male pupae develop faster than the female pupae.

The Adult

Emergence

At the early stages of brood emergence males are most abundant (Christophers 1960). When emergence is completed, the adult rests at the sides of the container for a few hours until the wings and the exoskeleton harden and darken (Nelson 1986). Additionally, the males have to rotate their genitalia 180^{o} into the right position (Nelson 1986, Becker 2003). This may last up to 24 hours.

Mating

Approximately 24 hours after emergence, mating takes place. Mating takes place with the female at rest or in flight (Schoof 1967). The males are attracted to the females due to the sound that is made by their wing beat. Females can begin to produce the desirable wing beat 2.5 hours after emergence (Roth 1948, Nelson 1968). The attracted male clasps the tip of the female abdomen with his genitalia and inserts his aedeagus into the female genital chamber. The duration of the copulation is brief and lasts less than a minute (Roth 1948). Females mate once since one insemination is enough to fertilize all the eggs that a female will develop in her

lifetime. Males on the other hand were shown to mate up to 10 and 15 times (Schoof 1967). After mating is complete the female searches for a blood meal. Once blood fed the female no longer emanates the wing beat tone (Roth 1948, Nelson 1968).

Feeding

The male mouthparts are not adapted for blood feeding. They meet their energy requirements by feeding on flower nectar. Females also feed on flower nectar to satisfy their carbohydrate needs. The female requires additionally a protein rich blood meal in order to be able to develop viable eggs (Magnarelli 1979, Clements 1992). The females of *Aedes aegypti* show preference in feeding on humans, a behavior known as "anthropophilic" (Carpenter and LaCasse 1955); however, they will feed on most vertebrates when available. Female mosquitoes use several stimuli to detect and reach their host. Carbon dioxide, octenol and lactic acid are some of the most documented host attractants (Acree et. al 1968, Takken and Kline 1989, Mboera and Takken 1997). Female mosquitoes fly upwind following the odors and other attractants released by the host. Once they are in close proximity to the host they use visual cues to locate the host. It was shown that *Ae. aegypti* are more attracted to black surfaces (Brett 1938) and to black-white interfaced surfaces (Brown 1966). As they approach even closer, temperature and other skin emissions guide them to the proper feeding site. Blood feeding usually takes place during daylight (Nelson 1986).

Flight range

Males fly less than the females (Nelson 1986). A female *Aedes aegypti* more commonly remains at the location where it emerged. In an experiment done by Trips and Hausermann (1986) it was shown that most marked *Ae. aegypti* were caught in the house in which they were released. When needed, a female can fly up to 2.5 kilometers in search of breeding sites

(Wolfinsohn and Galun 1953). It has been estimated than on average one female does not exceed 50 meters of flying during its life time (Nelson 1986).

Resting behavior

The most suitable resting place is a dark, quiet place. They mostly prefer to rest beneath and inside structures and rarely choose to rest outdoors on vegetation (Schoof 1967, Nelson 1986). They generally show preference resting on vertical surfaces.

Longevity

Aedes aegytpi adults in a laboratory setting can live for several months varying from 131 up to 225 days (Christophers 1960). However, in nature they usually survive for only a few weeks. Previous work has shown an average life-spam of 15 d for female mosquitoes outdoors (Nelson 1986). It is estimated that, when a population emerges, 50% of the adults die on average during the first week and 95% of the population dies after the first month. However, if the beginning emerging population is large, the subsequent older population will be adequately large to transmit disease and initiate an epidemic (Nelson 1986).

Fecundity

After a complete blood meal (2-3 mg), a female will produce and oviposit ~100 eggs (Nelson 1986). Smaller meals result in the production of small batches of eggs. It takes three days between blood engorgement and egg oviposition. It is also worth mentioning that a female can feed again the same day that oviposition took place. A single female can produce several egg batches in its life-time.

Public Health Importance of the Yellow Fever Mosquito

Aedes aegytpi is the main vector of 2 serious and life-threatening diseases, yellow fever, and the two forms of dengue, dengue fever (DF) and dengue hemorrhagic fever (DHF). Both diseases are caused by viruses in the family Flaviridae (Mullen and Durden 2002).

Yellow fever. It is caused by the YF virus. The relationship between Ae. aegytpi and YF virus was confirmed through the work of Carlos Finley (1881) and Walter Reed (1900). This discovery was of great importance, and it was the initiation of serious mosquito control measures to eradicate the mosquito vector, which brought great results and decrease significantly the vector populations. Currently, yellow fever is a serious threat in Central America, South America and lowland equatorial Africa. Yellow fever is the cause of approximately 30,000 deaths every year (Tabachnick 2004). The latest epidemic in the United States was in 1905 in New Orleans, where there were 3,402 cases and 452 deaths (Mullen and Durden 2002). Yellow fever is a hemorrhagic disease. Symptoms start to appear 3-6 days after infection. There are several cases of yellow fever with mild or no symptoms at all (Shroyer 2004).

Dengue fever (DF) and dengue hemorrhagic fever (DHF). Dengue is caused by the DEN virus that exists in 4 different and distinct serotypes (DEN-1, DEN-2, DEN-3, DEN-4). There are two forms of disease, the classic dengue fever and the most severe form the dengue hemorrhagic fever. Some of the symptoms of dengue are fever, headache, rash, and pain in the muscles and joints (Mullen and Durden 2002). The symptoms of the disease can vary from mild to fatal. The severity of the symptoms depends on the age as well as the infection history of the patient. Children show higher fatalities (CDC 2005). The first epidemic of DF that was reported occurred in 1779-1780 in three different continents simultaneously: Asia, Africa, and North America (CDC 2005). Dengue is responsible for hundreds of thousands of cases every year (CDC 2005). Specifically, from 1956 to 1980 there were 715,238 cases of DF and 21,345 deaths reported, and from 1986 to 1990 there were 1,263,321 cases and 15,940 deaths (Rigau-Perez et al. 1994).Currently, this disease is a problem to all tropical and subtropical areas of the world.

Indigenous transmission of the disease in the USA was reported in the years of 1980, 1986, and 1995 in Texas (Rigau-Perez et al. 1994, Mullen and Durden 2002).

Control Methods of the Yellow Fever Mosquito

Every organized mosquito control program is composed of 3 main components: surveillance of the mosquito target, methods for controlling the immature mosquito stages and methods for controlling the adult mosquitoes.

Surveillance

Surveillance is the basis of every pest control program. Constant knowledge of the distribution and composition of mosquito populations is the key to a well organized and effective control program. Also, pre- and post-treatment surveillance is necessary in order to evaluate the success of every control method implemented. There are various tools and methods available to monitor mosquito populations. When still in the immature stages the most common monitoring method is the dipping technique using a standard dipper, a dipper with a screened bottom or a cooking buster (Schreiber 2004). There are some monitoring techniques modified specifically for monitoring *Ae. aegypti* larvae. Harrison et al. (1982) and Undeen & Becnel (1994) developed 2 different types of floating traps specialized for collecting *Ae. aegytpi* larvae. When in the adult stage the surveillance of the mosquito populations is accomplished in two main ways: through the human landing rate technique and through the use of trapping devices. The most commonly used trap is the dry ice baited CDC trap with or without ice (Schreiber 2004). The New Jersey light trap is also used; however, when used in urban settings where *Ae. aegypti* are predominantly found, the lights from the houses will compete with the trap light source resulting in smaller numbers of mosquitoes captured (Schreiber 2004). Fay (1968) designed a trap, called the Fay trap, for specifically collecting *Ae. aegypti* adults. The Fay trap is similar to the CDC trap except that it is painted shiny black with the light source replaced by a glossy black board.

Methods for Controlling Immature Mosquitoes

The different methods available for controlling the immature mosquito stages are applied directly in the water and they can have larviciding action, pupiciding action, or they can even kill the adult mosquito while it is emerging. Some common methods for controlling immature mosquitoes are source reduction, use of the mosquito fish, *Gambusia affinis,* which is a form of biological control, use of bacterial insecticides such as *Bacillus thuringiensis israelensis* (B.t.i.) and *Bacillus sphaericus* (B.sph.), use of insect growth regulators (IGR's) such as chitin synthesis inhibitors and juvenile hormone analogues, use of surface control agents such as oils and monomolecular films, and use of insecticides such as the organophosphate insecticide temephos. Source reduction is one of the most effective methods for controlling container breeding mosquitoes such as *Ae. aegytpi*. Gubler et al. (1991) pointed out that "the only truly effective way to control mosquito vectors of dengue is source reduction". The mosquito fish was used in Malaysia in water containers for the control of *Ae. aegypti* (Becker et al. 2003). B.t.i. and B.sph. are two different species of naturally occurring soil bacteria capable of producing, during their sporulation, proteins that are toxic to mosquito larvae. The larvae need to be actively feeding on the bacterial spores in order for the product to be effective. B.t.i and B.sph. are available in different formulations such as liquids, powders, granules, tablets and briquets. B.t.i. is more effective in controlling *Aedes* and *Psorophora* species (Weinzierl et al 2005). B.sph. is effective in controlling *Culex*, *Psorophora* and *Culiseta* species (Weinzierl et al 2005). Its effectiveness in controlling *Aedes* species varies, for example it is not as effective in controlling *Ae. aegypti* populations. It was shown that *Ae. aegypti* larvae were 100 times less susceptible to B.sph. compared to other mosquito species (Becker 2003). A distinct difference between B.t.i. and B.sph. is environmental persistence. B.sph. can persist in the environment whereas B.t.i. has little residual activity. A new tablet formulation of B.t.i. and B.sph. was successfully used to control

Cx. p. pipiens and *Ae. aegypti* (Becker et al. 1991, Kroeger et al. 1995). Timing of application for both bacterial species is very critical, because early first and late fourth instar larvae do not feed and thus they will not receive the chemical. Diflubenzuron, a chitin synthesis inhibitor, interferes with the molting process of the larva and prevents the normal development of the cuticle (Becker et al. 2003). Methoprene is an analog of a naturally occurring insect hormone called juvenile hormone. Methoprene works by interfering with the mosquito's life-cycle. By doing so it prevents the insect's metamorphosis from an immature to an adult and causes adult sterility. Methoprene gets absorbed on contact through the larval integument, thus larvae don't need to be feeding in order for methoprene to act effectively. Methoprene is commercially available with the name Altosid. Altosid products come in different formulations such as liquids, powders, granules and briquets. Altosid formulations are known for their long residual activity for up to 150 days (Florida Coordinating Council on Mosquito Control 1998). Some commonly used surface agents are the Golden Bear oil and the monomolecular films Arosurf MSF and Agnique MMF. Surface oils cause mortality to mosquito larvae and pupae through suffocation because the oily surface prevents the insects from obtaining air through their siphon. On the other hand the monomolecular films prevent the insects from remaining on the surface of the water by reducing the tension of the water surface. Under these conditions larvae and pupae die from exhaustion as they use up their energy reserves trying to stay at the surface. Temephos is a heterocyclic organophosphate and is widely known with the commercial name Abate. It is available in different formulations such as liquids and granules. Temephos is very effective against all mosquito species and has a very low mammalian toxicity with an LD50 of 2030 mg/kg. It acts by inhibiting the activity of acetylcholinesterase enzyme in the Central Nervous System (CNS) synapses resulting in the accumulation of acetylcholine at its post-synaptic receptor. The excess

of acetylcholine causes neuroexcitation, rapid twitching of the muscles, and final paralysis of the insect. Temephos has been used successfully to control *Ae. aegytpi*. For example, in Thailand an up to 95.4% reduction of adult density was achieved after applying temephos 1% granule formulation on the bodies of water containing larvae (Gratz 1993, Becker et al. 2003). However, resistance to temephos has been reported (Grandes and Sagrado 1988) and is a serious concern.

Methods for Controlling Adult Mosquitoes

Chemical control of adult mosquitoes, commonly known as "adulticiding", is divided in two main categories based on behavioral traits of the mosquito: Control of the resting adults, which are residual applications referred to as barrier or surface sprays, and control of the flying adults, which are Ultra Low Volume applications referred to as space sprays. These two categories differ in the type of insecticides that are utilized as well in the application techniques that are used to distribute the insecticides on the target insect. Additionally, there is also one less popular approach available for controlling adult mosquitoes, which involves the use of vapor toxicants.

For the control of the resting adults, also, known as barrier treatment applications, residual insecticides are applied to perimeters around private residencies and recreational areas where mosquitoes are anticipated to rest. Some of the commonly used insecticides are deltamethrin, bifenthrin, betacyfluthrin, and lambda-cyhalothrin. Barrier treatments are large droplet applications, commonly applied during daylight hours, and are anticipated to last from a week up to two months depending on the insecticide used. Reiter (1991) pointed out that resting behavior of *Ae.aegytpi* plays a key role on the control of the insect, because unlikely most mosquito species, *Ae. aegytpi* prefer to rest inside (endophilic behavior) or around houses, and therefore they are hard to target through space-spraying applications. In agreement to Reiter's theory,

Chadee (1990) found that residual house spraying, a surface spray, was more effective for controlling *Ae. aegytpi* compared to ULV (ultra low volume applications) space spray.

The control of the flying adults is the most visible type of treatment with immediate results, and is the method of choice when there is a need for rapid reduction of mosquito populations like in the case of a disease outbreak. For this type of treatment the target is the flying adult mosquito and therefore the timing of spraying must coincide with mosquito flight activity. The treatments can be applied either aerially or by ground. The application technique is called Ultra Low Volume (ULV). ULV technology; as defined by the Environmental Protection Agency, is a method of dispensing insecticide in volumes less than 5 liters per hectare. Within mosquito control concentrate insecticide is often applied, therefore the output volume can be even lower < 1 liter per hectar. In other words ULV is a technique that applies the minimum amount of liquid of insecticides per unit area. The size of the droplets within the insecticidal cloud plays a very important role in determining the effectiveness of every spraying mission. Previous research has shown that the optimum droplet size for adult mosquito control is 5-10 microns (volume median diameter) for ground applications and 10-25 microns (volume median diameter) for aerial applications (Mount 1970). The size of the droplet determines the number of droplets per unit volume of insecticide, the time of which a droplet remains airborne, and the chances of the droplet penetrating through obstacles such as vegetation to reach the mosquito target (Becker 2003). Some common insecticides that have been used for controlling adult mosquitoes are fenthion, malathion and naled of the organophosphate family, sumithrin and resmethrin of the first generation synthetic pyrethroids and permethrin of the second generation synthetic pyrethroids (Florida Coordinating Council on Mosquito Control 1998). There has been a certain degree of failure of space spraying applications in controlling *Ae. aegypti* adults (Fox

1980, Perich et al. 1990, Gratz 1993) and a suggested explanation to that is their tendency to rest indoors (Becker 2003).

One last approach for adult mosquito control involves the use of slow release vapor toxicants. This is one of the least popular control methods and there has only been little research conducted to test the effectiveness of such applications. This could likely be attributed to the lack of insecticidal compounds with effective vapor toxicities. Dichlorvos (DDVP) is the one insecticide that has been most studied as a vapor toxicant against mosquitoes and other medically important pests (Maddock et al. 1963, Brooks & Schoof 1964, Brooks et al. 1965). Dichlorvos is an organophosphate insecticide and for the first time it was registered to be used as an insecticide in 1948 (EPA Pesticide Fact Sheet, 1978). A very common slow vapor release formulation of dichlorvos is resin strips. Slow release formulations of dichlorvos were shown to work effectively as an additional mosquito control method in occupied houses for malaria eradication programs (Mathis et al. 1959, Quarterman et al. 1963). However, the high acute mammalian toxicity of dichlorvos, in combination to reported resistance incidents has limited the use of dichlorvos as a widespread mosquito control method. Therefore, there is a need for new insecticidal compounds, with good vapor toxicities and novel modes of action that will replace dichlorvos. This research evaluated vapor toxicity of novel, low molecular weight, highly volatile formate, acetate, and heterobicyclic compounds on mosquitoes.

CHAPTER 3
LITERATURE REVIEW: THE HOUSE FLY

> And there came a grievous swarm of flies into the house of Pharaoh, and into his servant's houses, and into all the land of Egypt, and the land was corrupted by this kind of flies. (The Bible, Exodus 8:24, p. 74)

Classification and Distribution

The house fly *Musca domestica* (L.) belongs to the class Insecta, the order Diptera, suborder Cyclorrapha, and family Muscidae. It is commonly named house fly due to its close association to human settlements and activities. It is the most common fly in and around the home and it is a nuisance in every place where domestic animals are kept and waste accumulates. It is distributed around the world (West 1951) with the only exception of the Arctic, the Antarctic and areas of extreme high altitudes (Scott & Littig 1964). There are four different subspecies*: M. d. domestica* Linnaeus, *M. d. vicina* Macqvart, *M. d. nebulo* Fabricius, and *M. d. curviforceps* Sacca & Rivosecchi. The first three subspecies are found in temperate zones all over the world including subarctic and subtropical areas where as the fourth subspecies is limited to Africa (Keiding 1986).

Morphology

The Egg

They are 1-1.2 mm in length, banana shaped and creamy in color (West 1951, Keiding 1986).

The Larva

The larval stage is divided in three instars, from which the third one or else known as prepupa can reach up to 13 mm in length (Keiding 1986). Each instar is characterized by a cylindrical body divided in 13 well-defined segments with no appendages (West 1951). The larval head has no eyes and is located on the anterior, conical-shaped end of the larval body. For

feeding and for locomotion the larva has one strong and one small interior mouth hook located at the head. The posterior end of its body is rounded and consists of a pair of sclerotized structures, the spiracles, which are essential for breathing.

The Pupa

When the fly is ready for pupation, the integument of the third larval instar contracts and hardens to form a barrel shaped puparium (West 1951, Keiding 1986). The size of an average puparium is 6.3 mm in length (West 1951). For the first couple of hours the puparium is soft with a whitish, creamy coloration. As the cuticle hardens the color gradually darkens into a dark brown coloration.

The Adult

An adult house fly is approximately 6-9 mm in length and has a grayish coloration (West 1951, Mullen & Durden 2002). It has a pair of wings longer than the abdomen and when in rest they are directed posteriorly giving a triangular appearance to the fly (West 1951). The house fly's body is divided into three well defined regions: the head, the thorax, and the abdomen. The head has a pair of prominent eyes, where in the case of males are joined together (holoptic), and in the case of females are divided (dichoptic) (Mullen & Durden 2002). Adults have a pair of sucking mouthparts called the proboscis, which is composed of the labium that encloses the labrum and the hypopharynx and terminates in a two lobed labella (West 1951). The thorax is usually characterized by 4 dark, longitudinal stripes called vitae (Mullen & Durden 2002).

Life Cycle

The Egg

The eggs are laid in clusters in moist substrates of decaying, fermenting or putrefying organic matter (Schoof et al. 1954). One house fly can lay approximately 100-150 eggs (West 1951). The most favorable breeding sites are human waste and animal manure (Keiding 1986).

The eggs are very dependent upon moisture. It was shown that below 90% RH egg mortality increases (Keiding1986). Also, temperature plays an important role in the egg development. At 35^0C it takes 6-8 hours from oviposition to hatching. Below 13^0C and above 42^0C the eggs die before hatching (West 1951).

The Larva

The larval stage is divided into three instars. The first, second, and part of the third instar are called the feeding stages. They mainly feed on bacteria and their decomposition products. Odors attract the feeding stages to the breeding media. The larval stages tend to avoid light and prefer to occur in humid environments with a temperature around 35^0C (Keiding 1986). The late third instar is called prepupa and does not feed. In this stage the prepupae migrate to cooler and less humid environments where pupation takes place. There are several factors that affect the duration of the larval development such as nutrition, moisture, and temperature. Under optimal conditions it takes a minimum of 3-4 days for the completion of the larval development (Keiding 1986, Hogsette 1995).

The Pupa

The duration of this stage depends on humidity and temperature and lasts minimum of 3-4 days under optimal conditions (35-40C, 90% RH). The pupae have the ability to withstand lower humidity than the larvae. It has been shown that below 75% some pupae die and below 40% few survive (Keiding 1986).

The Adult

Emergence

When the development of the adult is completed within the pupal case, the adult brakes through the puparium and emerges quickly. The newly emerged adults are light grey and soft in appearance. Also, they have no wings. Before the newly emerged adults become fully capable of

flying they go through a phase that lasts several hours during which the cuticle hardens and the wings unfold (Keiding 1986). The young adults are ready for feeding 2-24 hours after emergence.

Mating

Males and females are ready for mating approximately 24 and 30 h, respectively, after emergence at optimal environmental conditions (Keiding 1986). Visual and olfactory stimulants are involved in the attraction between male and female adult flies (Colwell & Shorey 1977, Keiding 1986). A sex pheromone, (Z)-9- tricosene (muscalure), is produced by the females to attract the males (Carlson et al. 1971, Carlson & Leibold 1981). Also, another pheromone produced by the males is known to attract virgin females (Schlein & Galun 1984). Last, the wing beat frequency of the males was shown to have an effect on the mating behavior of the females (Colwell & Shorey 1976). Females usually mate once during their lifetime (monogamous) and store the sperm into the spermatheca (Keiding 1986), as opposed to males that can mate multiple times (polygamous).

Oviposition

Oviposition is closely dependent on air temperature. Below 15^0C no oviposition occurs (Keiding 1986, West 1951). Ammonia, carbon dioxide and other odors of rotting and fermenting materials attract the gravid females to their breeding medium (West 1956, Keiding 1986). Favorable breeding media include dung (Haines 1955), garbage and waste from food processing facilities (Schoof et al. 1954), sewage and accumulation of plant material (Silverly & Schoof 1955). The eggs are very sensitive to moisture and in order to be protected from desiccation are laid beneath the surface, within cracks and crevices. On average a female oviposits 120 eggs per batch (West 1951).

Feeding

House flies are considered to be polyphagous species, which means that they can feed on a wide variety of food material and they do not depend on particular types of proteins like other members of the family Muscidae (West 1951). Both male and female houseflies need water and sugars in order to survive. It is only the female flies that need additional protein in order to be able to develop viable eggs. They acquire their nutrients mostly from animal dung, human food and garbage. They are attracted to the food source mostly by visual cues. Odorous stimulants play some role when the food source is in close proximity (Keiding 1965). Flies are attracted to smells of fermenting and decomposing materials. When in contact with the food the fly uses special receptors on the legs and antennae to taste the food.

Longevity

Under laboratory conditions adult house flies can live up to a month (Keiding 1986). However, in field conditions the life span is considered to be less, approximately 2 weeks under ordinary conditions (West 1951).

Fecundity

A single female house fly produces approximately 120 eggs per cycle. The number of generations per year varies depending on the environmental conditions. At temperate climates house flies can produce up to 30 generations per year whereas in tropical climates the number of generations decreases to 10 per year (Keiding 1986). Theoretically, if a female fly laid 120 eggs in the middle of April, she would be responsible for the emergence of 5,598,720,000,000 flies in the middle of July (West 1951)!

Flight-range

House flies are strong fliers, can move forward at a rate of 6-8 km per hour, and don't tend to migrate (Keiding 1986). Provided that food and breeding medium is available they will remain

within a radius of 100-500 m from their breeding site. However, they have been shown to migrate up to 5-20 km from their breeding site (Schoof 1959, Keiding 1986, Nazni et al. 2005).

Public Health Importance of the House Fly

House flies, because of their behavior and biology can act as very effective disease vectors. They prefer to spend most of their life time on animal manure, human excrements, garbage and any type of decaying organic matter. However, they will eagerly utilize any other food source on any type of human facility that is available to them, and when that happens they will transfer pathogens from one substrate to the other. Houseflies are capable of transmitting pathogenic microorganisms through different modes of transmission. They can mechanically transfer them on the hair of their body (West 1951, Graczyk et al. 2005). They regurgitate them in their vomit, and they can also transfer them in feces through their alimentary track (Sulaiman et al. 2000). The pathogens transferred on the surface of the fly do not multiply, and they can only survive for a few hours. On the other hand, the pathogens in the alimentary track can multiply and survive longer for up to several days (West 1951). Therefore this mode of transmission is the most important and dangerous one.

The diseases that house flies transmit are intestinal diseases, eye diseases, and skin and wound diseases. Some examples of intestinal diseases are bacterial infections (shingellosis, salmonelosis, cholera), protozoan infections, and viral infections (poliomyelitis) (Levine & Levine 1991, Healing 1995, Mian et al. 2002, Graczyk et al. 2005). Outbreaks of diarrheal diseases in predominantly developing countries have been associated with the seasonal increase in abundance of filth flies (Graczyk et al. 2001). For example, in Thailand the seasonal peak in fly populations coincides with outbreaks of cholera (Echeverria et al. 1983). Examples of eye diseases that can be transmitted by houseflies are trachoma and conjunctivitis (Forsey & Darougar 1981). Last, an example of a skin disease is habronemiasis, a horse disease (Foil &

Foil 1988). This disease involves the deposition of infective house fly larvae onto mucous membranes of preexisting skin lesions on the stomach of horses.

Control Methods of the House Fly

Surveillance Methods

For every successful pest control approach it is vital to obtain information on the density and species composition of the pest population prior to any treatment. Post-treatment surveillance is necessary as well in order to evaluate the success of the control measures that have been implemented. There are several devices available for housefly surveillance that are commonly known as fly-traps. Fly-traps utilize visual stimuli and/or chemical attractants to lure flies. These could be ultra violet (UV) light traps which act as electrocutors, sugar/pheromone (sex pheromone-muscalure) baited traps, as well as cards or strips coated with sticky material to capture flies. Traps will not measure the absolute number of flies in a population, rather they will give an index and, also, the effectiveness of these traps to capture flies depends on their location within a certain area, temperature, and the physiological condition of the flies (Keiding 1986). Traps besides being a monitoring tool are also used for control operations.

Control Methods

Sanitation

A very old English quote says “Kill a fly in July, you’ve just killed one fly. Kill a fly in June, they’ll be scarced soon. Kill a fly in May, you’ve kept thousands away” (retrieved from West 1951). Within these 2 lines lies the very essence of a successful fly control plan. Due to their high reproduction rates, housefly populations can increase rapidly within a small period of time. Preventing the population from building up would be the best approach for effective and long-term fly control. The way to achieve prevention is to eliminate the conditions that allow flies to breed and multiply. Some examples of ideal housefly breeding media, as has been

discussed above, are animal manure, human feces, and garbage. Proper disposal of animal manure, human feces, and garbage is the primary and most effective method to control houseflies. Pickens et al. (1967) recommended frequent, if not daily, removal of animal manure. Barnard (2003) suggests collecting and storing manure in cone-shape piles to reduce the available surface area to flies. He also suggests proper composting or covering the organic matter with plastic to minimize fly attractiveness. West (1951) suggests storage of manure, when frequent disposal is not feasible, within concrete pits that will be fly-tight. Regarding disposal of human feces a properly operating sewage processing plant is necessary for each city and town (West 1951). Last, regarding garbage handling and disposal, open dumps must be replaced with sanitary landfills. In these landfills garbage will be compacted daily and covered with 24 inches of soil to effectively eliminate fly breeding (Keiding 1986). Another approach for treating garbage in large cities is complete combustion at temperatures of 1,400 ^{0}F to 2,000 ^{0}F, which would completely destroy organic material and prevent flies form breeding (Scott & Littig 1964). In conclusion, environmental sanitation is the best, long-term solution to every housefly problem.

Chemical control

For those situations where the fly population has already increased dramatically and immediate control is required there are several chemical based approaches that one could follow, which involve the usage of insecticides. There are, mainly, six different types of insecticide applications for the control of houseflies: direct insecticide application to the breeding sites for larval control (larvicides), application of residual sprays on housefly resting sites, introducing toxic man-made resting sites (impregnated cords/strips), applying toxic baits, applying space sprays directly to fly aggregations, and, last applying vapor toxicants (Keidig 1986, Barnard 2003).

Larvicides are applied as spot treatments on a regular basis in those areas where fly larvae are breeding. Some insecticides that have been used as larvicides include the organophosphates diazinon, trichlorfon, and fenthion, and several pyrethroids such as cypermethrin, deltamethrin, and permethrin. The insecticides are applied in different formulations such as emulsions or suspensions to thoroughly wet the upper 10-15 cm of the breeding medium (Barnard 2003). This method of control, however, should only be considered as an alternative to sanitation, and most of the times as a poor alternative. One of the problems that appear from the use of larvicides is the mortality of natural predators and parasites of houseflies (Keiding 1986, Scott et al. 1991). It has been suggested that even if larvicides offer temporary control they may result in increase of the fly population by disrupting the biological regulation by naturally occurring predators. Two products that have been used for fly larvae control and don't appear to have any important adverse effects on non-target organisms are the insect growth regulators diflubenzuron and cyromazine (Keiding 1986).

Treating naturally occurring resting areas of houseflies with residual insecticides or even introducing insecticide treated resting sites (such as toxicant impregnated strips and cords) is another popular approach for fly control. These are low-pressure, spot treatments of residual insecticides on those surfaces that flies are anticipated to land and rest. Several examples of insecticides that have been used for this type of application are the organophosphates (dimethoate, trichlorfon and naled), and the pyrethroids (cypermethrin, permethrin, and deltamethrin) (Barnard 2003). The effectiveness of this method depends on the type of the insecticide used, the environmental conditions like sunlight exposure which accelerates the insecticide degradation, but mostly it depends on the right location of the treatment in time and space according to the resting behavior of the fly (Keiding 1965). Keiding (1965) in his review

on observations of the housefly behavior in relation to its control concluded firstly, that houseflies show preference for resting indoors at lower night temperatures (below 15-20 ^{0}C) and outdoors in warmer nights, secondly the upward movement of flies to the ceiling or branches of trees after sunset, and last the general preference of flies to rest on narrow objects, edges, and anything protruding from large surfaces. He suggested that since houseflies tend to have an aggregated night time distribution, control efforts should be mostly directed against the night resting sites.

Insecticides in the form of baits came into prominence in the early 1950's (Gahan et al. 1953). The first form of baits that were initially used for fly control contained simple sugar water or some other type of attractant combined with poisons such as sodium arsenite and formaldehyde. Since the development of modern insecticides, newer baits have been developed that can be divided into three main categories: dry scatter baits, liquid baits, and paint-on baits (Keiding 1986, Barnard 2003). The newer baits utilize a variety of organophosphate (i.e. dimethoate, malathion, naled, diazinon) and carbamate (i.e. propoxur, bendiocarb, methomyl) insecticides as active ingredients. The effectiveness of the baits to attract flies can be enhanced by the addition of attractants, such as the sex pheromone, muscalure. Baits can provide satisfactory control and reduce fly populations in short periods of time. However, they must be applied one to six times per week (Barnard 2003) in order to be effective. Also, they have the advantage that development of resistance is generally less compared to residual sprays. They must be kept, however, away from animals and children.

Both outdoor and indoor space treatments for housefly control involve the usage of mists or aerosols of insecticide solutions or emulsions that directly target aggregations of resting or flying adults. Space treatments do not provide long-term fly control but instead they provide a

temporary relief from housefly nuisance. Therefore they should be applied in those situations where excessive housefly nuisance is being observed, and they should be used as an additional tool and not as the main approach technique for controlling houseflies. For indoor applications, hand and power sprayers are used to apply the material. For the outdoor applications mist sprayers, thermal foggers, or even Ultra Low Volume (ULV) application methods can be used to disperse the material. For indoor treatments natural pyrethrins or synthetic pyrethroids would be the insecticide of choice, due to their ability to provide quick knockdown without presenting any toxic hazards (Schmidtmann 1981). Also, indoors space treatments must be applied during those times when most flies are aggregated indoors. For the outdoor treatments the application can take place both by ground and air (Mount, 1985) and has as ultimate goal to eliminate fly populations around those areas with high human activity such as recreational areas and food markets. For the outdoor treatments, in addition to the pyrethroids, several organophosphate compounds are used as well (i.e. malathion, naled, diazinon).

One last approach for fly control involves the use of slow release vapor toxicants. This has been one of the least popular control methods and there has been little research conducted to test the effectiveness of such an application. This could be attributed to the lack of insecticidal compounds with effective vapor toxicities. Dichlorvos (DDVP) is the one insecticide mostly studied as a vapor toxicant against house flies (Miles et al. 1962, Matthysse & McClain 1972). Dichlorvos is an organophosphate insecticide and for the first time it was registered to be used as an insecticide in 1948 (EPA 2006). A very common formulation of dichlorvos is in resin strips. The resin strips were shown to work effectively against adult flies in enclosed spaces. Resin strips were, also, proven effective for fly control within garbage cans or other similar receptacles that may not be fly-tight. The high mammalian toxicity of dichlorvos, in combination to reported

resistance incidents (Bailey et al. 1971) has limited the use of dichlorvos as a fly control approach. Therefore, there is a need for new insecticidal compounds, with good vapor toxicities and novel modes of action that will replace dichlorvos. This research evaluated vapor toxicity of novel, low molecular weight, highly volatile formate, acetate, and heterobicyclic compounds on house flies.

CHAPTER 4
LITERATURE REVIEW: NOVEL VOLATILE COMPOUNDS AND INSECTICIDE SELECTIVITY

Novel Volatile Compounds

Insecticides are divided into five categories according to their mode of action: physical poisons, protoplasmic poisons, metabolic inhibitors, neuroactive agents and stomach poisons (Matsumura 1980). Some insecticides have multiple modes of actions, as that seems to be the case with the novel compounds studied in this thesis project. Nguyen et al. (2007) studied toxicity, synergism and neurological effects of the novel formates, acetates, and heterobicyclics on *Drosophila*. *Drosophila* was chosen as representative of the order Diptera. According to their findings, the compounds possess a diverse range of activities and modes of actions, as they seem to act as both metabolic inhibitors and neuroactive agents. They were able to identify a role for cytochrome P450-based metabolism in activation and/or deactivation of the various heterobicyclics, esterase-based activation of some formate esters, and finally neurological action at chloride and sodium channels by the novel compounds.

Also, Haritos & Dojchinov (2003) studied a range of alkyl esters on beetles, in an attempt to discover the toxic agent of the alkyl esters within the insects. Their intentions were to determine whether it was the intact ester or one or more of its break down products that were responsible for the toxic effects. Their findings revealed esterase-based activation of the formate esters, which comes in agreement with Nguyen et al (2007). Haritos & Dojchinov (2003) showed that volatile formate esters were more toxic than other alkyl esters due to their hydrolysis to formic acid and its inhibition of cytochrome c oxidase. The process involves 3 main steps. First, a wide variety of many esterases hydrolyse the formate esters into formic acid and their corresponding alcohols. Then, formic acid binds to cytochrome a_3 and inhibits cytochrome c oxidase activity (Nicholls 1975). Last, the inhibition of cytochrome c oxidase prevents the

utilization of molecular oxygen by cells, leading to loss of cell function and subsequently cell death.

This research evaluated the vapor toxicity effects of the novel volatile compounds on two different insect species: the yellow fever mosquito and the common house fly. There is no work published to my knowledge regarding the mode of action of the novel volatile esters and heterobicyclcis on mosquitoes and house flies. This paper constitutes the first publication on the toxicity of the novel volatile compounds on mosquitoes and house flies.

It is very often that insecticides exhibit different toxicities among different insect species (Camp at al. 1969, Coats 1979, Mallipudi & Fukuto 1979). Understanding how various insecticides exhibit different toxicities among different insect species (insecticide selectivity) will be necessary in order to appropriately explain and discuss the results presented in Chapters 5 & 6 of this paper.

Insecticide Selectivity

Once the insecticide enters the insect body it is recognized as a foreign substance or "xenobiotic", and is metabolized to a less toxic and more polar substance that will eventually be removed from the body. This metabolic process is called "detoxicification". However, it has been shown that insecticides can also be converted into more toxic substances once within the insect body. This process is known as "activation" (Feyereisen 2005). By far the two most significant reactions involving the metabolism of insecticides are the NADPH-requiring cytochrome P450 mono-oxygenases and the esterases or hydrolases (Feyereisen 2005, Oakeshott et al. 2005). The first system is also known as the "mixed function oxidase" (MFO) system and it performs the first oxidative enzymatic attack on xenobiotic compounds. These enzymes are quite versatile and accept most xenobiotic copmpounds as their substrate. They require NADPH to deliver the electrons down an electron transport system with cytochrome P450 as the terminal

oxidase of the electron transport chain. The final product of this reaction is the oxidized form of the xenobiotic compound. The second reaction is a hydrolysis reaction and it involves the action of several hydrolases, such as carboxylesterases, amidases, type A-esterases, which split esteratic insecticide substrates with the addition of water to yield alcohols and acids as the final products.

The activity of these two enzymatic systems varies among different insect species, potentially resulting in species differences in susceptibility to various insecticidal compounds. Also, both enzymatic systems have been involved in insecticide resistance mechanisms. Following I have several examples that demonstrate how the activities of these 2 enzymatic systems vary among different insects and can affect the insect responses on various insecticides. Casida et al. (1976) reported different ability of esterases to hydrolize pyrethroid insecticides among 5 different insect species. Brooks (1986) reported esterases to be more important enzymes for pyrethroid detoxification in *Spodoptera littoralis* (the Egyptian cotton leafworm), *Trichoplusia ni* (cabbage looper) and *Chrysoperla carnea* (common green lacewing) larvae, and oxidases more important in *Tribolium castaneum* (red flour beetle) larvae. Claudianos et al. (2006) reported that honeybee shows much greater susceptibility to insecticides compared to *Anopheles gambiae* and *Drosophila* due to a deficit of detoxification enzymes: there are only about half as many cytochrome P450 monooxygenases and carboxyl/cholinesterases in the honeybee compared to *Anopheles gambiae* and *Drosophila*. Phillips at al. (1990) and Benedict et al. (1994) showed that genetically transformed Drosophila (op degrading gene) with high levels of organophosphate hydrolases shows over 20-fold greater paraoxon resistance compared to untransformed controls. Chang & Whalon (1987) showed that in resistant strains of predatory mites some esterase isozymes demonstrated higher rates of synthetic pyrethroid hydrolysis compared to the non-resistant strains. Also, P450 over expression was shown to various

insecticide resistant strains. For example Kasai et al. (1998) showed that the metabolism of permethrin to 4-hydroxypermethrin was higher in microsomes from Culex mosquito larvae resistant to permethrin than from the susceptible strain. Last, conversion of fipronil to its sulfone by P450 has a marginal effect on the toxicity of the parent chemical in Diabrotica virgifera (Scharf et al. 2000). However, in Blattela germanica it was shown that the oxidation of fipronil to its sulfone constitutes an activation step (Valles et al. 1997).

CHAPTER 5
EVALUATION OF VAPOR TOXICITY OF NOVEL LOW MOLECULAR WEIGHT COMPOUNDS ON MOSQUITOES

Introduction

Volatile insecticides have been commonly used as fumigants for the control of structural pests and the protection of agricultural commodities. However, they have been mostly ignored for the control of medical importance pests such as mosquitoes and flies. Dichlorvos (DDVP) is the one volatile insecticide studied mostly on mosquitoes and flies. Dichlorvos is an organophosphate insecticide and was registered in 1948 (EPA 2006). One very common formulation of dichlorvos is resin strips. Resin strips were initially registered for use in areas where flies, mosquitoes and other nuisance pests occur. Dichlorvos has been classified by the Environmental Protection Agency (EPA) as a "probable human carcinogen", and because of its implications in human health in 2006, its use in homes was restricted to confined spaces such as wardrobes, cupboards and closets (EPA Office 2006). Therefore, there is a need for replacement of dichlorvos with friendlier, less toxic chemistries. Highly volatile, low molecular weight formates, acetates, and heterobicyclics may be potential replacements for dichlorvos, and in their own right may offer a new class of chemistry.

Thirty novel, low molecular weight compounds with insecticidal activity were tested on *Drosophila melanogaster* Meig. (Scharf et al. 2006). The compounds belonged to six different families: heterobicyclics, formates, acetates, propionates, butyrates and valerates. *Drosophila* was used as a model to assess potential efficacy of these novel chemistries against mosquitoes and flies. Findings showed 7 highly effective compounds with vapor toxicity: four formate esters and three heterobicyclics. The reaction of an organic acid and an alcohol is called esterification, where the end products are always ester and water. Formate esters are organic compounds composed of formic acid and a corresponding alcohol. Acetate esters, similarly to formate esters,

are composed of acetic acid and a corresponding alcohol. On the other hand the structure of heterobicyclics is made from fused 5, 6-membered rings.

For my research I investigated the vapor toxicity effect of 4 heterobicyclic compounds, 7 formate, and 4 acetate esters directly on mosquitoes. Most of the compounds that I evaluated in the work presented here are naturally occurring products. They are found on fruits such as apples, bananas, strawberries, oranges, kumquats, and coconuts just to name a few. They are commercially used as flavoring agents in products such as coffee, chocolate, fruity drinks, rum, wine, and tobacco. Most of the compounds have a rather strong and fruity odor, and therefore they have many uses as odor agents. Another interesting characteristic of these products is that they are part of the chemical structure of some pharmaceutical drugs, responsible for treating insomnia, osteoporosis, and asthma. In Tables 5-1, 5-2, and 5-3 the chemical structures of each individual chemical can be seen. Information such as molecular weight, boiling point, density, natural occurrence and other physical properties are included in the same table as well.

Materials and Methods

Chemicals

Fifteen novel insecticides (Sigma Aldrich Chemical, Milwaukee, WI) were tested; 7 formate esters [ethylene glycol di-formate (EGDF), methyl formate, ethyl formate, propyl formate, butyl formate, hexyl formate and heptyl formate), 4 heterobicyclic esters (menthofuran, benzothiophene, coumaran and dimethyl-coumarone) and 4 acetate esters (propyl acetate, butyl acetate, pentyl acetate and hexyl acetate). Dichlorvos (DDVP) was tested as a positive control (Chem Service, West Chester, PA). All insecticides were >99% pure and in liquid form except for thiophene that came in a crystalline solid form. Insecticide stock solutions were prepared in acetone at concentrations of 2, 100, 150, 200, 300 and 400 µg/µl. All compounds and stock

solutions were held at –20°C in glass vials with rubber lined caps to prevent vapor escape, until placed in experiments.

The insecticide synergists SSS-tributyl-phosphorotrithioate (DEF) and piperonyl butoxide (PBO), which are esterase and cytochrome P450 inhibitors respectively, were used (Mobay Chemical Co., Kansas City, MO and MGK Inc., Minneapolis, MN). DEF and PBO were >95% pure. DEF and PBO stock solutions were prepared at 100 µg/ml in acetone.

Insects

Mosquitoes [USDA-CMAVE Orlando strain of *Aedes aegypti* (L.)] reared at the University of Florida in Gainesville were used. Mosquitoes were reared on a 12:12 (L: D) photoperiod, at 25°C and ~50% RH. Mosquito larvae were fed on a powder diet consisting of 2 parts liver (MB Biomedicals LLC, Aurora, OH) and 3 parts yeast (Modern Products Inc., Thiensville, WI). The diet was diluted in deionized water to a 40 g/liter concentration. Approximately 1,500 larvae were reared in plastic trays (53.3 by 40.6 cm) containing 3 liters of water. The quantity of the diluted diet varied depending on the larval instar. Mosquito larvae were not fed for 24 h after hatching. Second and third instars were fed 30 ml of the diluted medium per day; whereas, the diet of the fourth instars was decreased to 20 ml per day. When majority of pupation had occurred no more food was provided. Pupae were removed and placed into deli cups filled with deionized water. The deli cups were then placed into screened rearing cages (39.4 by 26.7 by 26.7 cm) for adult emergence. Mosquito adults were maintained on a 10% [w/v] solution of sugar water.

Prior to each treatment 3 to 5-d-old adult mosquitoes were aspirated from their cages and placed into plastic deli cups on ice until their activity was reduced. Ten females were removed from the deli cups using a feather tip forceps. A minimum of 300 mosquitoes were selected for exposure to each insecticide.

Bioassay

Main bioassay set-up. This bioassay was adapted from Scharf et al. (2006) and Nguyen et al. (2007) (Fig. 5-8). Ten females were transferred from the deli caps into 125 ml plastic vials. Caps with an opening of ~2.6 cm in diameter, covered with common fiberglass window screening (~1.55 mm mesh), were used to close the vials. The screening prevented insect escape while allowing for gas exchange. Along with the mosquitoes a cotton wick (~1.5 cm in length) dipped in 10% w/v solution of sugar water was placed in the vials. A toothpick (~6.3 cm in length) was used to support the wick. The wick was provided as the nutrient and moisture source. The mosquitoes were given 1 h to recover from the chilling effects of the ice prior to the treatment, and then every vial was placed into a Mason 1 liter (1 quart) glass jar along with an untreated filter paper (55 mm in diameter). Prior to closing the glass jar the filter paper was treated with the proper quantity of insecticidal solution using an eppendorf pipette. The concentrations of the insecticide solutions varied depending on the insecticide tested. Methyl and propyl formate were applied at a 100 µg/µl concentration and in a range from 1.2-1.8 mg. Coumaran, butyl formate, and hexyl formate were applied at a 150 µg/µl concentration and in a range from 0.75-3 mg, 1.05-1.95 mg, and 1.05-1.95 mg, respectively. Menthofuran, benzothiophene, ethyl formate, heptyl formate, EGDF, propyl acetate and butyl acetate were applied at a 200 µg/µl concentration and in a range from 2-4 mg, 1.6-4 mg, 1.4-2.6 mg, 1.6-4 mg, 2-4 mg, 2.8-4 mg and 2.8-4 mg, respectively. Dimethyl-coumarone and hexyl acetate were applied at a 400 µg/µl concentration and in a range from 2-8 mg. DDVP was applied at a 2 µg/µl concentration and in a range from 0.016-0.04 mg. There was, also, a blank control where the filter paper received no chemical at all, and a solvent control, which received a volume of acetone identical to the highest insecticide solution volume (up to 20 µl). The jars were closed rapidly and tightly to prevent vapor escape and after a 24 h exposure mortality was recorded. In

order to determine mortality the jars were shaken for a minimum of 15 sec before mosquito movement was observed. A mosquito was recorded dead when there was no movement observed.

Synergist (DEF and PBO) bioassay set-up. The effect of the synergists PBO and DEF for toxicity was investigated on three of the fifteen insecticides tested above: ethylene glycol di-formate, heptyl formate and menthofuran. The synergist bioassays were conducted in the same way described above except that an extra step was added. That extra step involved the exposure of the mosquitoes to the synergist, prior to their exposure to the insecticides (Nguyen et al. 2007). For the synergist bioassay the plastic vials were replaced with 125 ml glass vials to prevent absorption of the synergist into the plastic. Synergist stock solutions at 100 µl were pipetted to every glass vial, using an eppendorf pipette, so that every vial would contain 10 µg of the synergist. Previous studies have shown that this synergist quantity causes no mortality in Drosophila after 24 h of exposure (Nguyen et al. 2007). After treating the vials with the synergist the vials were rolled on their sides under a fume hood to ensure equal distribution of the synergist on the inner surfaces while the acetone evaporated. Once acetone evaporated, 10 mosquitoes were added in every glass vial along with a moist cotton wick, and were held for an hour to allow for the synergist to take effect. Along with the blank and the solvent control, a synergist control was added where the mosquitoes were only exposed to the synergist.

Data Analysis

In those cases where control mortality was observed data was adjusted using the Abbott's formula (Abbott 1925). When control mortality exceeded 10% that rep was discarded. Probit analysis was performed and the LC_{50} and LC_{90} of each insecticide with and without the synergist were estimated (SAS Institute 2003). The data reported in Tables 5-4, and 5-5 include slope, goodness of fit characteristics (chi-square, P-value) and LC_{50} and LC_{90} estimates with 95%

confidence limits. LC estimates with non overlapping 95% confidence limits were considered significantly different.

Body-weight corrected LC_{50s} of each insecticide for mosquitoes and *Drosophila* were calculated (Table 5-6, Fig. 5-7). One hundred individuals from both species were weighed and that weight was recorded. That number was then divided by 10 to give the average weight of 10 mosquitoes and 10 *Drosophila* (0.0153 and 0.006 g, respectively). The average weight of the 10 insects was used to adjust the LC_{50} from mg/liter into mg/ g of insect body weight/liter.

PoloPlus 2.0 (2005) was used to calculate the potency ratios of the LC50s with & without the synergist. The program calculated 95% confidence limits for every ratio. The 95% CI were used to determine whether there were significant differences in the LC50s due to the effect of the synergists (Table 5-5).

Linear regression analyses were performed (SAS Institute 2003) that compared LC_{50} estimates versus molecular weight, density and boiling point of the seven formate esters, the four heterobicyclics and the four acetate esters (Figs. 5-3, 5-4, 5-5, and 5-6).

Results

Toxicity Evaluation of Novel Compounds

DDVP was by far the most toxic compound tested on mosquitoes. Specifically, it was 54.4 times more toxic compared to the second best compound, the formate ester methyl formate. Within the novel compounds, overall, formate esters were the most toxic family followed by the heterobicyclics and, last, by the acetate esters (Table 5-4, Fig. 5-1).

Formate esters. Methyl formate was the most toxic ester (LC_{50} estimate 1.36 mg/liter), followed by butyl, propyl, ethyl, hexyl formate, EGDF, and heptyl formate. The toxicities of propyl and ethyl formate were not significantly different and there was no major difference between them and butyl formate. EGDF and heptyl formate (LC_{50} estimates 2.99 and 3.17

mg/liter, respectively) were the least toxic formate esters with toxicities in the same range as the heterobicyclics, the second best performing family of esters.

Heterobicyclics. Coumaran was the most toxic heterobicyclic (LC_{50} estimate 2.03 mg/liter), followed by benzothiophene, dimethyl-coumarone and menthofuran. Benzothiophene and dimethyl-coumarone were not significantly different. There were significant differences in the slopes among the 4 heterobicyclic compounds. Coumaran, the best performing heterobicyclic, had the smallest slope, which suggests that there is a lot of heterogeneity on the response of the insects to the insecticide. On the other hand, menthofuran, the heterobicyclic with the poorest performance, had the biggest slope, which suggests a lot of homogeneity on the response of the insects towards the insecticide.

Acetate esters. Hexyl acetate was the least toxic compound (LC_{50} estimate 5.09 mg/liter). The toxicities of propyl, butyl and pentyl acetate were not significantly different.

Toxicity Evaluation of Novel Compounds with the Synergistic Effect of DEF and PBO

Whne heptyl formate and EGDF were cotreated with DEF their toxicities decreased significantly (Table 5-5, Fig. 5-2). The toxicity of heptyl formate decreased by 1.35 times, where as the toxicity of EGDF decreased by 2.56 times. Also, when menthofuran was combined with PBO, its toxicity increased significantly.

Evaluation of the Role of Volatility in Toxicity

Molecular weight, density, and boiling point were investigated as predictors of volatility. A chemical with lower molecular weight (lighter chemical), lower boiling point and lower density volatilizes faster compared with a chemical having higher molecular weight (heavier chemical), higher boiling point and higher density. Regression analysis between toxicity and volatility predictors was performed for each of the three families separately and for all three families together (Figs. 5-3, 5-4, 5-5, and 5-6). For the formate esters the regressions of LC_{50} versus

molecular weight, boiling point, and density were correlated with R^2 0.57, 0.69, and 0.19, respectively. For the heterobicyclics the regression of LC_{50} versus molecular weight was correlated with R^2 0.88. On the other hand, the regressions of LC_{50} versus density and boiling point were weak ($R^2 < 0.25$). Last, for the acetate esters the regressions of LC_{50} versus molecular weight, density and boiling point were weak ($R^2 = 0.24$, $R^2 = 0.20$, $R^2 = 0.24$ respectively). When combined families regression was performed there was a poor correlation between toxicity and all 3 volatility predictors, except maybe with molecular weight ($R^2 = 0.5$).

Discussion

Comparing Toxicities of Novel Compounds Among Mosquitoes and *Drosophila*

My study is the first report of the toxic effects of the novel volatile formate, heterobicyclic, and acetate compounds on mosquitoes. Scharf et al. (2006) reported the toxicity of these novel compounds on *D. melanogaster* Meig. *Drospophila* was used as a model to assess potential efficacy of these compounds on mosquitoes and flies. Body-weight corrected LC_{50} values (in mg per g of insect per liter) of the 15 volatile compounds and the organophosphate DDVP on mosquitoes and *Drosophila* can be seen in Table 5-6 and Fig. 5-7. There were significant differences among the toxicities of the compounds on mosquitoes and *Drosophila*. DDVP was by far the most toxic insecticide for both insects and was significantly more toxic to mosquitoes than *Drosophila*. DDVP has been known as a very effective insecticide against various insects for many years. Maddock and Sedlack (1961) gave one of the earliest reports regarding the toxicity of DDVP on mosquitoes. They reported that 0.015 µg of DDVP per liter of air will give 100% kill of *Anopheles* mosquitoes. All compounds, except for menthofuran, were significantly more toxic to mosquitoes than *Drosophila*. On average the 14 compounds were approximately 3.5 times more toxic to mosquitoes, whereas menthofuran was 1.7 times more toxic to *Drosophila*. On mosquitoes there was a toxicity trend observed among the three families, with

formates showing overall higher toxicity, followed by heterobicyclics and last by acetates. However, there was not an apparent trend on toxicity among the three ester families on *Drosophila*. Some of the best performing compounds and some of the poorest ones belonged to the same chemical families. It was only the acetate esters that consistently showed poor toxicity. The best 7 performing insecticides with vapor toxicity on *Drosophila* were the two heterobicyclics menthofuran and benzothiophene. These two compounds were followed by the formate esters butyl, hexyl, heptyl formate, the heterobicyclic coumaran and the formate ester ethyl formate. The best 7 performing compounds on mosquitoes were the formate esters methyl, butyl, propyl and ethyl formate. These compounds were followed by the formate ester hexyl formate, the heterobicyclics coumaran, and benzothiophene. What is interesting is that the most toxic compound on *Drosophila,* menthofuran, is one of the least toxic compounds when tested on mosquitoes. Conversely, the most toxic compound on mosquitoes, methyl formate, is one of two least toxic compounds when tested on *Drosophila*.

There are several possible explanations as to why the compounds performed differently on mosquitoes than *Drosophila*. A first explanation could be differences on the insect handling techniques during the experimentation. Scharf et al. (2006) used CO_2 to knock down *Drosophila* prior to exposing them on the insecticides, whereas I used ice for knocking down mosquitoes. Another explanation could be physiological differences in acquiring and metabolizing insecticides. The lethal effects of insecticidal compounds depend upon the amount of insecticide that reaches the target site (site of action). The amount of insecticide that reaches the target site is controlled by certain processes such as penetration through the insect cuticle, diffusion through the insect spiracles, bioactivation/biodegradation within the insect body, travel distance to the site of action and finally excretion just to name a few (Quraishi 1977, Matsumura 1980, Yu

2006). Different insect species can differ greatly in susceptibility to insecticidal compounds due to distinct differences in the physical and physiological processes mentioned above. Insecticides with high vapor pressures, such as the insecticides studied in this paper, show the tendency to enter the insect body through the spiracles (Matsumura 1980). Therefore, the susceptibility of an insect to a vapor toxicant is believed to be correlated with its rate of respiration (Vincent & Lindgren 1965). In general, different insects exhibit different patterns of gas exchange when at rest (Lighton 1988, 1990, Lighton & Berrigan 1995). The most familiar and well studied pattern is the Discontinuous Gas Exchange Cycle (DGC) (Kestler 1985, Lighton 1994), where the spiracles remain closed for lengthy periods of time allowing for no gas exchange. This closed-spiracle phase is followed by a fluttering-spiracle phase and finally an open-spiracle phase where the accumulated CO_2 escapes form the tracheal system to the surrounding environment. Little information, however, is available on the respiratory pattern of small insects (body weight ~ 1 mg) such as *Drosophila* (Williams et al.1997, Williams and Bradley 1998, Lehman et al. 2000, Fielden et al. 2001), and even less information is available on mosquitoes (Diarra et al. 1999, Gray & Bradley 2003, Gray & Bradley 2006). What is known so far is that both mosquitoes & *Drosophila* have the ability to control gas release from their tracheal system. There is some evidence to support that both insects perform DGC, however further research is needed to conclusively test this hypothesis. Due to the absence of evidence one should consider that mosquitoes and *Drosophila* may follow a different breathing pattern. If this is true, the insects may be allowing different amounts of insecticide to enter their body and this may be one of the factors responsible for the different responses they show to the various insecticides tested.

Another explanation could be differences in the detoxification systems among mosquitoes and *Drosophila*. Once an insecticide enters the insect body it is perceived as a foreign substance

or xenobiotic, and is metabolized by different metabolical processes with the ultimate goal to be converted into a less toxic polar substance that will eventually be removed form the body. This metabolic process is called “detoxification”. By far the two most significant reactions involving the metabolism of insecticides are the NADPH-requiring general oxidation system and the hydrolysis of esters (Matsumura 1980). The activity of these two enzymatic systems varies among different insect species, resulting in species differences in susceptibility to various insecticidal compounds (Casida et al. 1976. Brooks 1986, Valles et al. 1997, Scharf et al. 2000).

Implications of the Synergistic Effects of PBO and DEF on the Toxicity of the Novel Compounds on Mosquitoes

The modes of action of the novel formate, acetate, and heterobicyclic compounds on mosquitoes so far remain undefined. According to the results presented in this study there seems to be a significant effect of cytochrome P450 enzymes on menthofuran detoxification. Also, there was evidence supporting esterase-based activation of both heptyl formate and ethylene glycol di-formate. When heptyl formate and ethylene glycol di-formate were synergized with the esterase inhibitor DEF their toxicities decreased by 1.35 and 2.56 times, respectively. The first finding comes in agreement with Nguyen (2007), who showed in Drosophila that P450 enzymes play a significant role in menthofuran detoxification and activation, depending on the fly strain. The second finding comes in agreement with both Haritos & Dojchinov (2003), and Nguyen (2007), who supported esterase based activation of some formate esters. In order for more legitimate conclusions to be made more extensive and complete research needs to be conducted where all of the esters will be tested in combination with both synergists on susceptible and even resistant mosquito species.

Structure-activity Relationships of the Three Families of Novel Compounds

As one might expect the tendency of a chemical to volatilize should play an important role in its vapor-phase toxicity. However, this did not always seem to be the case with the novel, volatile compounds studied in this research. Scharf et al (2006) did a combined ester and heterobicyclic regression analysis between toxicity and the three volatility predictors: molecular weight, boiling point, and density. According to their findings there was a statistically weak correlation between toxicity and all three volatility predictors. I performed regression analyses for each of the three families separately and for all of them combined together. When studied each family separately I was able to show reasonable correlation between toxicity and volatility predictors for some of the families. For the formate esters the regression analysis demonstrated a reasonable correlation between toxicity and the volatility predictors: molecular weight, and boiling point. However, there was a weak correlation between toxicity and density. For the heterobicyclics there was a strong correlation between toxicity and molecular weight, and a weak correlation between toxicity and the rest two volatility predictors (boiling point, density). For the acetate esters there was overall a weak correlation between the three volatility predictors and toxicity. When I evaluated all the compounds together there was overall a statistically weak correlation between toxicity and volatility. My findings, as well as Scharf's et al. (2006) findings, showed that high ester volatility did not necessarily coincide with high ester toxicity. What that implies is that there should be other factors, such as structure dependent factors, affecting the widely varying toxicity of the volatile esters.

With respect to the heterobicyclics 2 structure-activity relationship trends are apparent. First, when no peripheral methyl groups are present, oxygen in the first position of the furan ring is associated with greater toxicity than if sulfur is in this position (i.e. coumaran > benzothiophene). However, that contradicts Scharf's et al (2006) findings, where they showed

that sulfur in the first position of the furan ring is associated with higher toxicity. Second, when oxygen is in the first position of the furan ring and peripheral methyl branches are present, adjacent methyl branches are associated with greater toxicity than opposing methyl branches (i.e. dimethyl-coumarone > menthofuran). This finding comes in disagreement with Scharf et al. (2006), who showed that opposing methyl branches are associated with higher toxicity than adjacent methyl branches. The compounds showed different structure-activity relationships between mosquitoes and *Drosophila*, which suggests that the compounds may follow different metabolic pathways and may exhibit different modes of action within the two insect species.

With respect to the formate and acetate esters some structure activity relationships are apparent as well. First, as the aliphatic chain length on the acid group increases toxicity decreases for the majority of the formates (i.e. methyl formate>ethyl formate=propyl formate>hexyl formate>heptyl formate). On the other hand, there was a different activity-structure trend for formates when tested on *Drosophila* (Scharf et al. 2006). They showed that esters of intermediate chain length demonstrated greater toxicity (i.e butyl formate, hexyl formate), with lower toxicity for methyl and ethyl formates. Also, formates elicited higher toxicity than acetates implying that the formate group within the aliphatic chain is correlated with higher toxicity than the acetate group. This comes in agreement with Scharf et al. (2006), who showed that acetate esters were a less toxic family compared to formate esters.

In conclusion, DDVP was by far the most toxic insecticide for both insects and was significantly more toxic to mosquitoes than *Drosophila*. All insecticidal compounds, except for menthofuran, were significantly more toxic to mosquitoes than *Drosophila*. On mosquitoes there was a toxicity trend observed among the three families, with formates showing overall higher

toxicity, followed by heterobicyclics and last by acetates. The novel compound with the highest insecticide activity on mosquitoes was methyl formate.

Table 5-1. Physical and chemical properties of formate esters

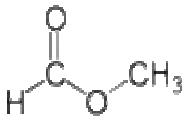
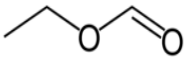
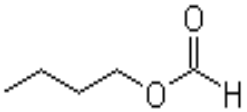
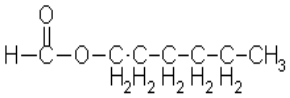
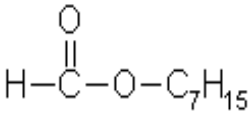
Formate Esters	Mol. Weight	bp (^{0}C)	Density (g/ml)	Natural occurrence	Used as	Other Properties
Methyl formate 	60.05	33	0.974	–	-Quick drying finishes -Alternative to sulfur dioxide in domestic refrigerators	Clear liquid with an ethereal odor
Ethyl formate 	74.08	53	0.921	–	Flavoring agent (raspberries flavor)	Characteristic smell of rum
Propyl formate 	88.11	80.5	0.904	Apple, Pineapple, Plum, Currant	Flavoring agent(brandy & rum products)	Colorless liquid with a sweet fruity/berry odor
Butyl formate 	102.13	106.5	0.892	Pear	Flavoring/odor agent (rum, pear, plum products)	Colorless liquid with a fruity/green odor
Hexyl formate 	130.18	155.5	0.879	Pear	Flavoring/odor agent (apple, banana, lemon, strawberry, orange products)	Colorless liquid with a medium fruity odor
Heptyl formate 	144.21	178	0.882	Kumquat	Flavoring/odor agent (apple, apricot, coconut, kumquat,peach, rose, wine products)	Colorless liquid with a medium green/floral/ apple scent
Ethylene glycol di-formate 	118.09	176	1.226	–	–	Colorless, odorless liquid

Table 5-2. Physical and chemical properties of heterobicyclics

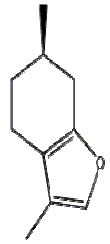
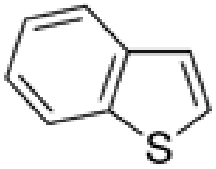
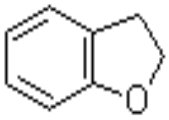
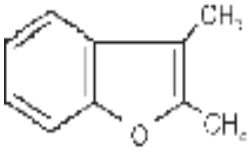
Heterobicyclic Esters	Mol. Weight	bp (^{0}C)	Density (g/ml)	Natural occurrence	Used as	Other Properties
Menthofuran 	150.22	205	0.97	Peppermint oil	Flavoring/odor agent (chocolate, coffee, peppermint)	Bluish clear liquid with a musty nutty/coffee odor
benzothiophene 	134.20	221.5	1.149	Constituent of petroleum related deposits (lignite tar)	Found in the chemical structure of pharmaceutical drugs for treating osteoporosis & asthma (raloxifen, zileuton)	Solid crystalline form with an odor similar to naphthalene
Coumaran 	120.15	188.5	1.065	_	Found in the chemical structure of pharmaceutical drugs (insomnia treatments)	_
Dimethyl-coumarone 	146.19	101.5	1.034	Cade oil Tobacco Coffee	Flavoring/odor agent (chocolate, coffee, tobacco, vanilla, leather products)	Pale yellow liquid with a strong phenolic odor

Table 5-3. Physical and chemical properties of acetate esters

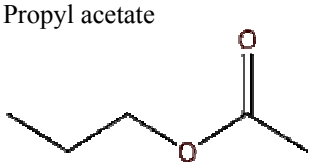
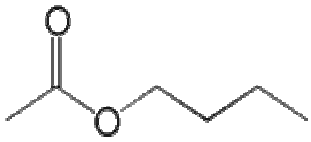
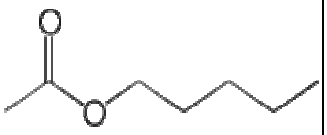
Acetate Esters	Mol. Weight	bp (^{0}C)	Density (g/ml)	Natural occurrence	Used as	Other Properties
Propyl acetate 	102.3	102	0.888	_	Flavoring/odor agent	Clear colorless liquid with an odor of pears
Butyl acetate 	116.16	125	0.88	Several fuits (eg. Apples in the Red Delicious variety)	Flavoring agent (candy, ice-cream, cheeses, baked goods)	Colorless liquid with a fruity odor
Pentyl acetate 	130.18	146	0.876	_	_	Colorless liquid with an odor similar to banana odor
Hexyl acetate 	144.21	169	0.87	_	Flavoring and fragrance agent	Colorless liquid with a fruity/pear odor

Table 5-4. Vapor toxicities of 15 novel, low molecular weight, volatile compounds and the organophosphate DDVP to mosquitoes *Aedes aegypti* (L.)

Insecticide Families Insecticides	Slope ± SE	LC50 mg/liter (95% CI)	LC90 mg/liter (95% CI)	$\chi 2$	P
Organophosphates					
DDVPa	4.84 ± 0.51	0.025 (0.023-0.027)	0.047 (0.042-0.056)	4.50	0.11
Formate esters					
Methyl formate	9.84 ± 1.10	1.36 (1.311-1.40)	1.83 (1.74-1.98)	4.09	0.13
Ethyl formate	9.12 ± 0.82	1.7 (1.64-1.78)	2.37 (2.25-2.54)	3.06	0.22
Propyl formate	7.87 ± 1.70	1.69 (1.62-1.80)	2.45 (2.15-3.38)	2.66	0.10
Butyl formate	7.79 ± 0.76	1.54 (1.48-1.60)	2.25 (2.08-2.50)	4.50	0.10
Hexyl formate	7.52 ± 0.90	1.86 (1.77-2.00)	2.76 (2.47-3.29)	4.00	0.13
Heptyl formate	4.79 ± 0.55	3.17 (2.92-3.51)	5.88 (4.99-7.54)	4.56	0.33
EGDF	9.12 ± 0.81	2.99 (2.89-3.11)	4.14 (3.90-4.48)	1.98	0.96
Heterobicyclics					
Menthofuran	11.66 ± 1.72	3.62 (3.51-3.73)	4.66 (4.37-5.21)	2.36	0.49
Benzothiophene	4.83 ± 0.50	2.89 (2.71-3.10)	5.33 (4.70-6.42)	1.20	0.54
Dimethyl-coumarone	7.86 ± 0.49	2.98 (2.88-3.09)	4.35 (4.13-4.62)	4.94	0.55
Coumaran	3.14 ± 0.34	2.03 (1.84-2.26)	5.19 (4.22-7.05)	2.57	0.27
Acetate esters					
Propyl acetate	5.89 ± 1.22	4.31 (3.98-5.11)	7.11 (5.73-11.8)	0.24	0.88
Butyl acetate	7.83 ± 1.22	3.91 (3.73-4.21)	5.70 (5.04-7.16)	0.03	0.98
Pentyl acetate	8.04 ± 1.21	3.80 (3.65-4.05)	5.49 (4.91-6.72)	0.24	0.88
Hexyl acetate	6.15 ± 0.69	5.09 (4.75-5.41)	8.23 (7.52-9.38)	0.63	0.42

[a] Positive Control.

Table 5-5. Vapor toxicity of EGDF, heptyl formate & menthofuran with and without the synergistic effect of DEF and PBO to mosquitoes *Aedes aegytpi* (L.)

Insecticides	Slope ± SE	LC50 mg/liter (95%CI)	LC90 mg/liter (95% CI)	$\chi 2$	P	Potency ratio a
EGDF	9.12 ± 0.81	2.99 (2.89-3.11)	4.14 (3.90-4.48)	1.98	0.96	
EGDF + DEF	6.36 ± 0.69	7.67 (7.23-8.08)	12.19 (11.19-13.80)	0.34	0.98	2.56 (2.39-2.73)
Heptyl formate	4.79 ± 0.55	3.17 (2.92-3.51)	5.88 (4.99-7.54)	4.56	0.33	
Heptyl formate + DEF	8.33 ± 0.82	4.29 (4.12-4.47)	6.12 (5.74-6.70)	2.20	0.69	1.35 (1.23-1.49)
Menthofuran	11.66 ± 1.72	3.62 (3.51-3.73)	4.66 (4.37-5.21)	2.37	0.49	
Menthofuran + PBO	8.47 ± 0.96	3.37 (3.24-3.52)	4.77 (4.39-5.42)	5.37	0.14	0.932 (0.89-0.98)

[a] LC_{50}+synergist / $LC_{50.}$

Table 5-6. Body-weight corrected vapor toxicities of 15 novel, low molecular weight, volatile compounds and the organophosphate DDVP to mosquitoes *Aedes aegytpi* (L.) and *Drosophila melanogaster* Meig.

Insecticide Families Insecticides	Mosquito LC50 mg/gr of insect/liter (95%CI)	Drosophila LC50 mg/gr of insect/liter (95%CI) c
Organophosphates		
DDVPa	1.68 (1.5-1.76)	3.7 (3.2-4.3)
Formates esters		
Methyl formate	88 (85.68-91.5)	824 (636.6-1,776)
Ethyl formate	112 (107.2-116.3)	550 (493.3-636.6)
Propyl formate	110 (105.8-117.6)	610 (593-626.6)
Butyl formate	100 (96.7-104.5)	304 (266.6-332)
Hexyl formate	130 (115.6-130.7)	380 (356.6-400)
Heptyl formate	206 (190.8-229.4)	450 (420-475.3)
EGDF	194 (188.8-203.3)	834 (676.6-1,846)
Heterobicyclics		
Menthofuran	230 (229.4-243.7)	136 (120-150)
Benzothiophene	190 (177.1-202.6)	266 (236.6-293.3)
Dimethyl coumarone	194 (188.2-201.9)	654 (513.3-817.3)
Coumaran	132 (120.3-147.7)	490 (446.6-553.3)
Acetate esters		
Propyl acetate	282 (260.1-333.9)	683 (ND)b
Butyl acetate	256 (243.8-275.2)	607 (576.6-643.3)
Pentyl acetate	248 (238.5-264.7)	597 (550-660)
Hexyl acetate	333 (310.4-353.6)	553 (526.6-583.3)

[a] Positive control.
[b] Not determined.
[c] *Drosophila* data Scharf et al. 2006.

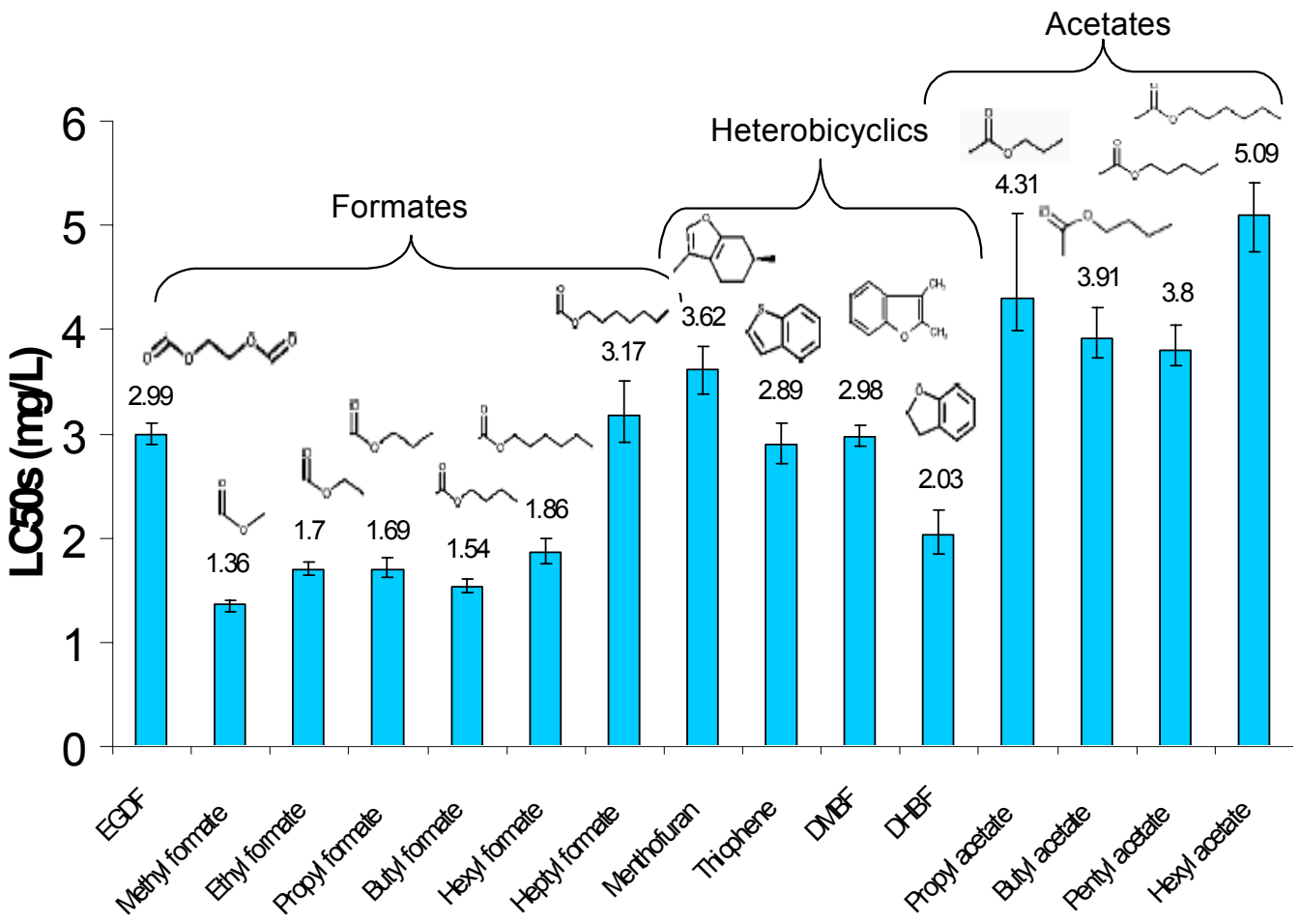


Figure 5-1. The LC_{50} values of mosquitoes *Aedes aegypti* (L.) when exposed on vapors of 15, novel, low molecular weight compounds.

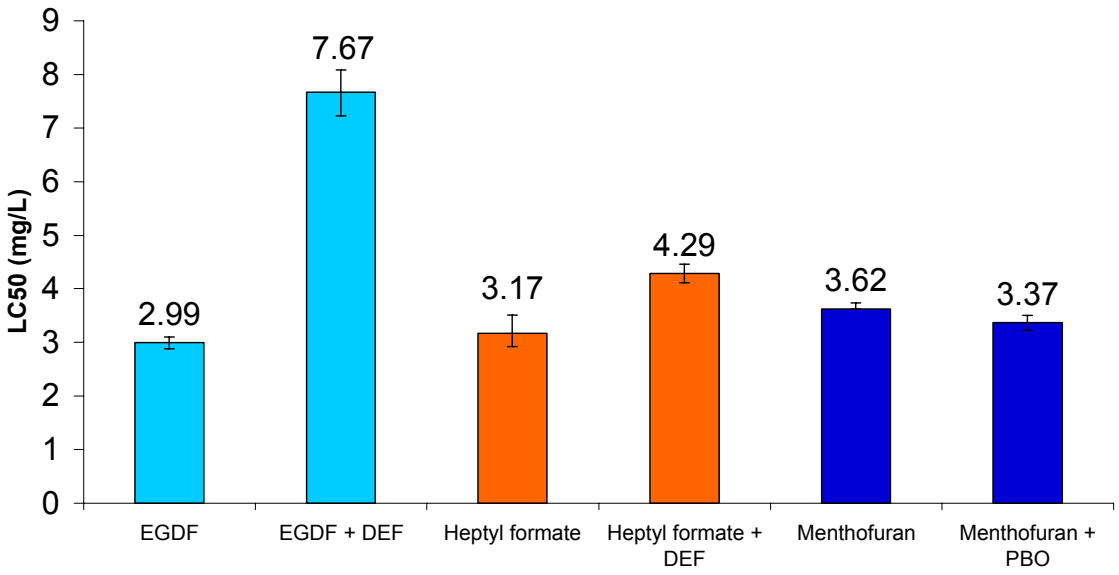
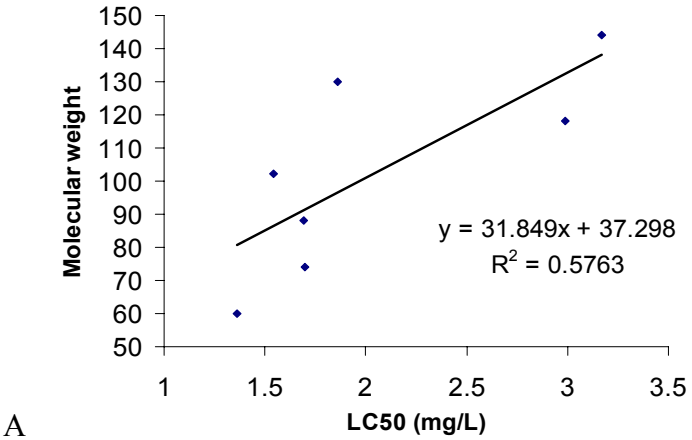
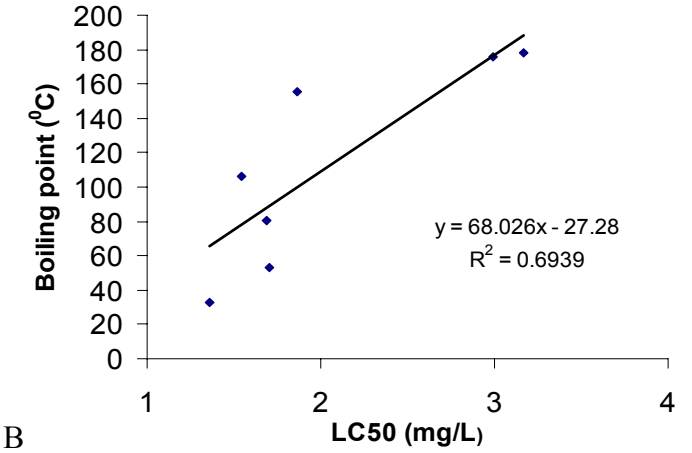


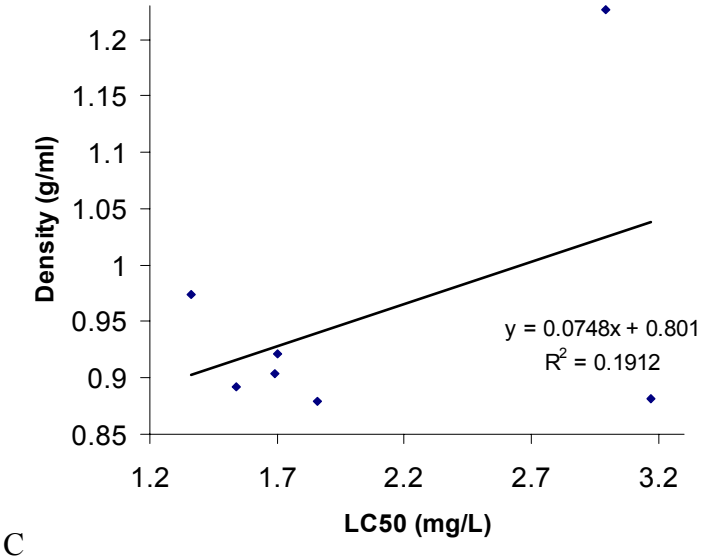
Figure 5-2. The LC_{50} values of mosquitoes *Aedes aegypti* (L.) when exposed on the vapors of EGDF, heptyl formate, and menthofuran with and without the synergistic effect of DEF and PBO.



A

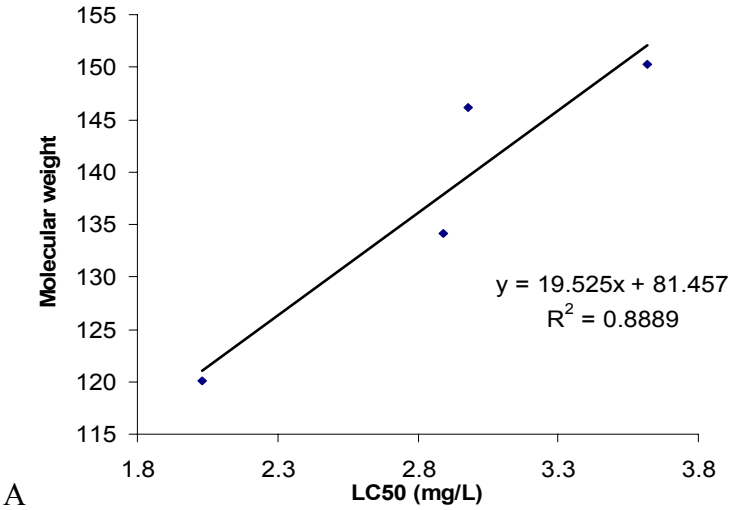


B



C

Figure 5-3. Regression analyses of the LC_{50} versus the physical properties of each of the 7 formate esters. A) LC_{50} versus molecular weight. B) LC_{50} versus boiling point. C) LC_{50} versus density.



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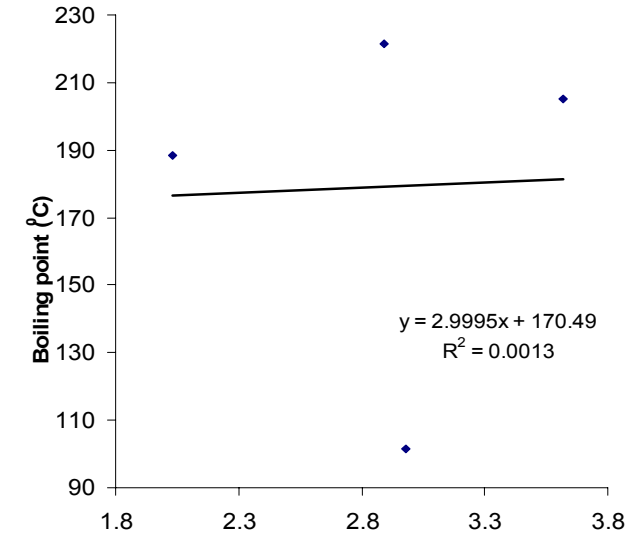

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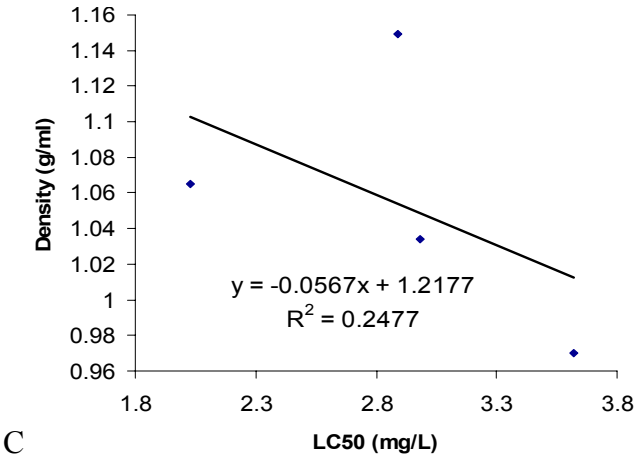
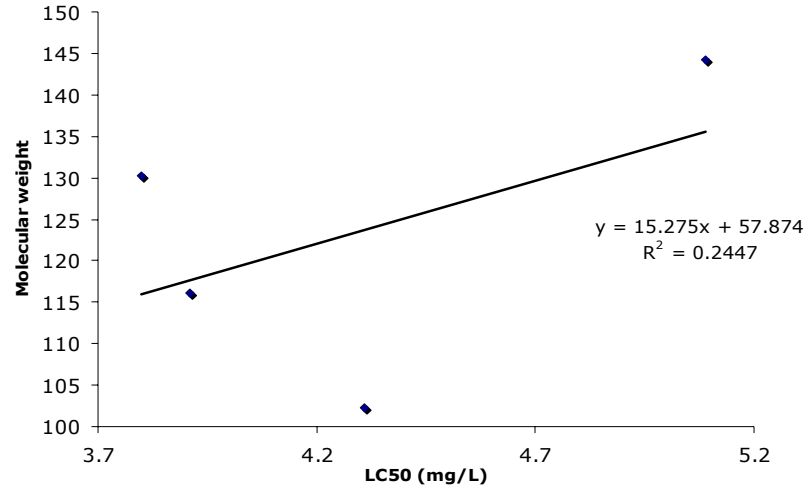


C

Figure 5-4. Regression analyses of the LC_{50} versus the physical properties of each of the 4 heterobicyclics. A) LC_{50} versus molecular weight. B) LC_{50} versus boiling point. C) LC_{50} versus density.



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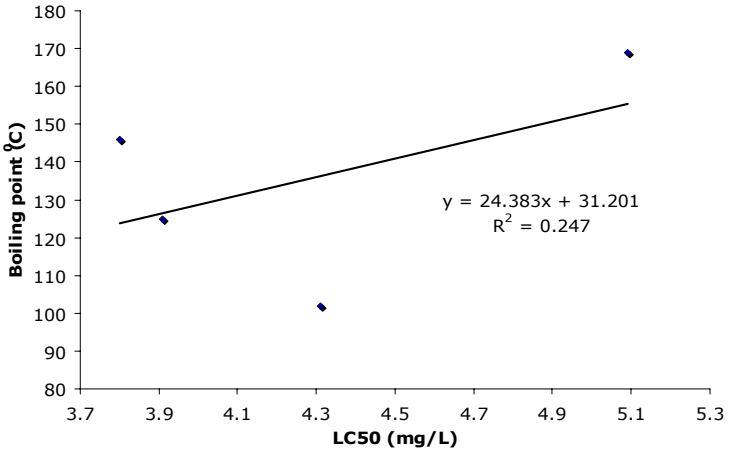

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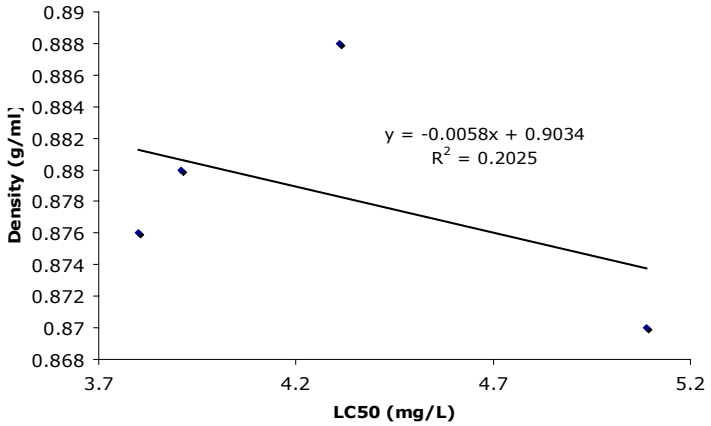
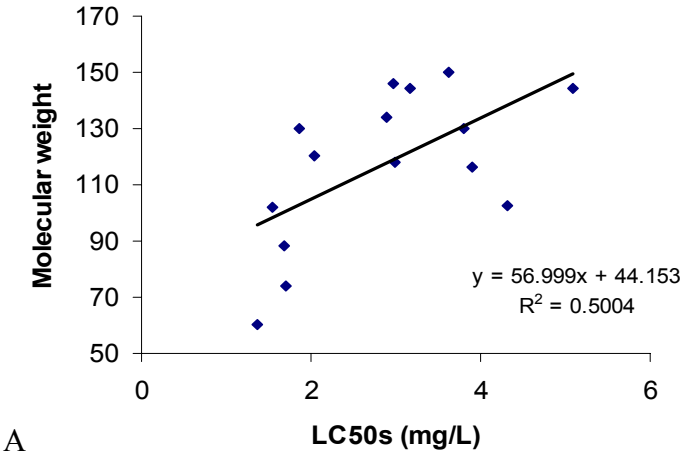
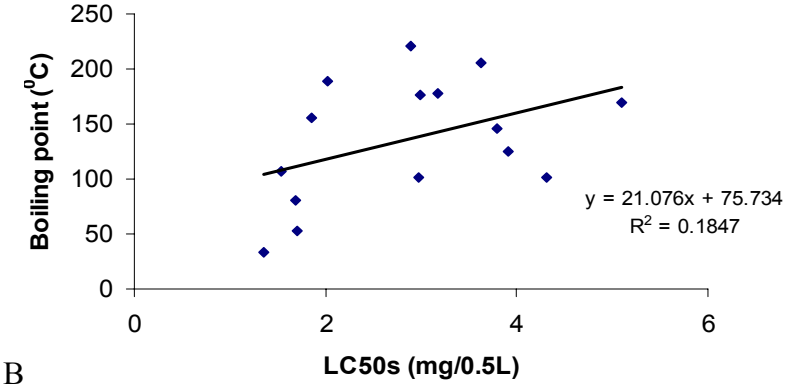


C

Figure 5-5. Regression analyses of the LC_{50} versus the physical properties of each of the 4 acetates. A) LC_{50} versus molecular weight. B) LC_{50} versus boiling point. C) LC_{50} versus density.



A



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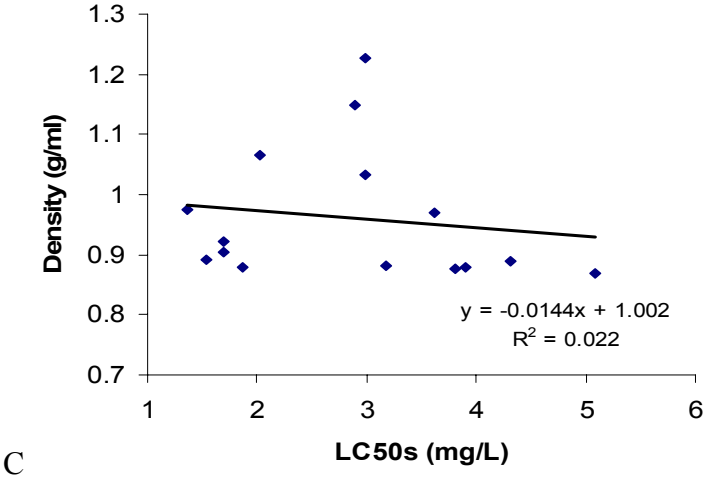

C

Figure 5-6. Regression analyses of the LC_{50} versus the physical properties of all the 15 novel compounds (formates, acetates, and heterobicyclics). A) LC_{50} versus molecular weight. B) LC_{50} versus boiling point. C) LC_{50} versus density.

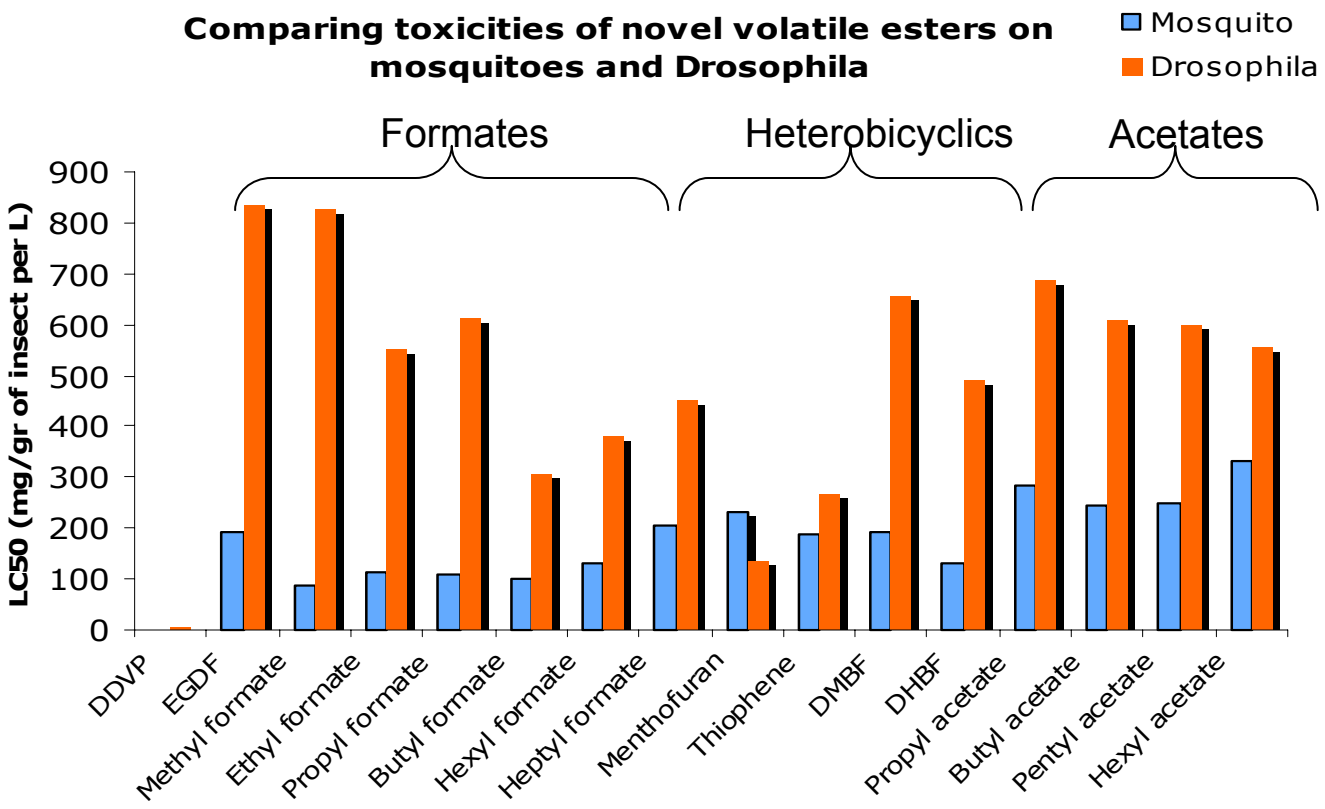

Figure 5-7. Body-weight corrected LC_{50} values for mosquiotes *Aedes aegypti* & *Drosophila* when exposed to the vapors of the 15 low molecular weight esters and the organophosphate DDVP.

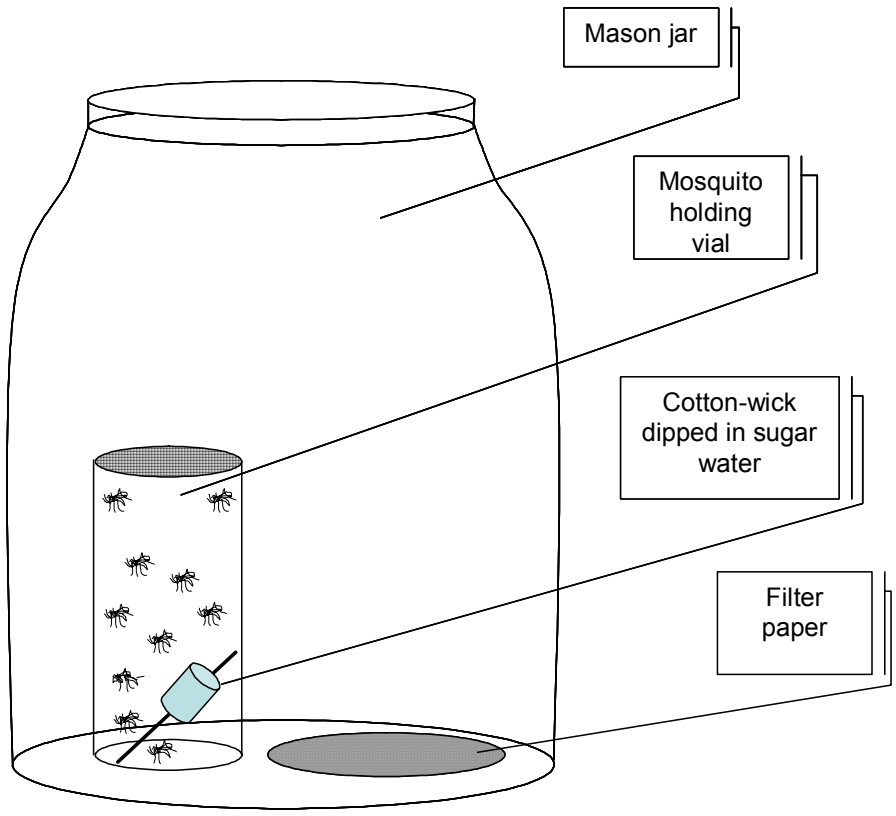

Figure 5-8. Main bioassay set-up.

CHAPTER 6
EVALUATION OF VAPOR TOXICITY OF NOVEL LOW MOLECULAR WEIGHT COMPOUNDS ON HOUSE FLIES

Introduction

Volatile insecticides have been commonly used as fumigants for the control of structural pests and the protection of agricultural properties. However, they have been mostly ignored for the control of medical importance pests such as mosquitoes and flies. Dichlorvos (DDVP) is the one volatile insecticide mostly studied on mosquitoes and flies. Dichlorvos is an organophosphate insecticide and was registered in 1948 (EPA 2006). One very common formulation of dichlorvos is resin strips. Resin strips were initially registered for use in areas where flies, mosquitoes and other nuisance pests occur. Dichlorvos has been classified by the Environmental Protection Agency (EPA) as a "probable human carcinogen", and because of its implications in human health in 2006, its use in homes was restricted to confined spaces such as wardrobes, cupboards and closets (EPA Office 2006). Therefore, there is a need for replacement of dichlorvos with friendlier, less toxic chemistries. Low molecular weight, highly volatile formates, acetates, and heterobicyclics may be potential replacement for dichlorvos.

Thirty novel, low molecular weight compounds with insecticidal activity were tested on *Drosophila melanogaster* Meig. (Scharf et al. 2006). The compounds belonged to six different families: heterobicyclics, formates, acetates, propionates, butyrates and valerates. *Drosophila* was used as a model to assess potential efficacy of these novel chemistries against mosquitoes and flies. Findings showed 7 highly effective compounds with vapor toxicity: four formate esters and three heterobicyclics. The reaction of an organic acid and an alcohol is called esterification, where the end products are always ester and water. Formate esters are organic compounds composed of formic acid and a corresponding alcohol. Acetate esters, similarly to formate esters,

are composed of acetic acid and a corresponding alcohol. On the other hand the structure of heterobicyclics is made from fused 5, 6-membered rings.

For my research I investigated the vapor toxicity effect of three of those compounds, one heterobicyclic (menthofuran) and two formate esters (ethylene glycol diformate, heptyl formate) directly on house flies. Both heptyl formate and menthofuran are naturally occurring compounds. Heptyl formate is naturally found in kumquats and has a floral, apple scent. It is commercially used as a flavoring agent in apple, apricot, kumquat, and wine products to name a few. Menthofuran is naturally found in peppermint oil. It has a musty, nutty odor and is used as a flavoring/odor agent in coffee and chocolate products. In Table 5-1 the chemical structures of the three chemicals can be seen. Information such as molecular weight, boiling point, density, natural occurrence and other physical properties are included in the same table as well.

Materials and Methods

Chemicals

Three novel insecticides (Sigma Aldrich Chemical, Milwaukee, WI) were tested; one heterobicyclic (menthofuran) and 2 formate esters [heptyl-formate and ethylene glycol di-formate (EGDF)]. Dichlorvos (DDVP) was tested as a positive control (Chem Service, West Chester, PA). All insecticides were >99% pure and in a liquid form. Insecticide stock solutions were prepared in acetone at concentrations of 200 or 10 µg/µl. All compounds and stock solutions were held at –20^{o}C in glass vials with rubber lined caps to prevent vapor escape until placed in experiments.

The insecticide synergists SSS-tributyl-phosphorotrithioate (DEF) and piperonyl butoxide (PBO), which are esterase and cytochrome P450 inhibitors respectively, were used (Mobay Chemical Co., Kansas City, MO and MGK Inc., Minneapolis, MN). DEF and PBO were >95% pure. DEF and PBO stock solutions were prepared at 100µg/ml in acetone.

Ceramic Rods

Hydrophilic, ceramic, porous rods (Small Parts, Inc., Miami, FL) were used to provide controlled vapor release of the volatile compound heptyl formate. The rods were 7.5 cm in length and 1.3 cm in diameter. The porous size of the ceramic rods was 2.5 microns and 38% of each rod was void volume. In order to decrease insecticidal release rate, the rods were covered tightly with aluminum foil leaving one end exposed, prior to being treated with insecticide.

Insects

Flies [Horse-Teaching-Unit (HTU) strain of *Musca domestica* (L.)] reared at the University of Florida in Gainesville were used. Flies were reared on a 12:12 (L:D) photoperiod at 26^{o}C and ~55% RH. Fly larvae were fed on a medium containing 3 liters wheat bran, 1.5 liters water, and 250 ml of dairy calf feed (Calf Manna; Manna Pro. Corp., St. Louis, MO) pellets. Fly pupae were separated from the medium and placed into screened rearing cages (40.6 by 26.7 by 26.7 cm) for emergence. Fly adults were maintained on a 2 parts granulated sugar and 1 part powdered milk diet with water *ad libitum*.

Prior to each treatment 3 to 5-d-old adult flies were aspirated from their cages and placed into plastic deli cups on ice until their activity was reduced. Ten females were removed from the deli cups using a feather tip forceps. A minimum of 300 flies were selected for exposure to each insecticide.

Bioassay

Main bioassay set-up. Ten females were transferred from the deli caps into 125 ml plastic vials. Caps with an opening of ~2.6 cm in diameter, covered with common fiberglass window screening (~1.55 mm mesh), were used to close the vials. The screening prevented insect escape while allowing for gas exchange. Along with the house flies a cotton wick (~1.5 cm in length) dipped in 10% w/v solution of sugar water was placed in the vials. A toothpick (~6.3 cm in

length) was used to support the wick. The wick was provided as the nutrient and moisture source. The flies were given 1 h to recover from the chilling effects of the ice prior to the treatment, and then every vial was placed into a Mason 1 liter (1 quart) glass jar along with an untreated filter paper (55mm in diameter). Prior to closing the glass jar the filter paper was treated with the proper quantity of insecticidal solution using an eppendorf pipette. The concentration of the insecticidal solution varied depending on the insecticide tested. Menthofuran was applied at a 200 µg/µl concentration and in a range from 1-4 mg. Pure heptyl formate and pure EGDF were applied in a range from 18-44 mg and 2.5-15 mg, respectively. DDVP was applied at a concentration of 10 µg/µl and in a range from 0.1-0.2 mg. There was also a blank control where the filter paper received no chemical at all, and a solvent control, which received a volume of acetone identical to the highest insecticide solution volume (up to 20 µl). In order to determine mortality the jars were shaken for a minimum of 15 sec before fly movement was observed. A fly was recorded dead when there was no movement observed.

Synergist (DEF and PBO) bioassay set-up. The effect of synergists on the toxicity of the three insecticides tested above was investigated. The synergist bioassay was conducted in the same way described above except that an extra step was added. That extra step involved the exposure of the house flies to the synergist, prior to their exposure on the insecticides. For the synergist bioassay the plastic vials were replaced with 125 ml glass vials to prevent absorption of the synergist into the plastic. Synergist stock solutions at 100 µl were pipetted to every glass vial using an eppendorf pipette, so that every vial would contain 10 µg of the synergist. Previous studies have shown that this synergist quantity causes no insect mortality after 24 h of exposure (Nguyen et al. 2007). After treating the vials with the synergist, the vials were rolled on their sides under a fume hood to ensure equal distribution of the synergist on the inner surfaces while

the acetone evaporated. Once acetone evaporated, 10 house flies were added in every glass vial along with the moist cotton wick and were held for an hour to allow for the synergist to take effect. Along with the blank and the solvent control, a synergist control was added where the flies were only exposed to the synergist.

Controlled vapor release of heptyl formate. Ceramic rods were used to determine effectiveness of heptyl formate in killing house flies over time. For the rod bioassay the house flies were handled the exact same way as described before. Three different treatments were tested and one blank control. In the first treatment flies within the glass jars were exposed to a single rod embedded with 3.81 g of heptyl formate. This treatment was replicated five times. In the second treatment flies were exposed to a filter paper embedded with 0.95 g of heptyl formate, which is the amount of heptyl formate that a single rod is anticipated to release within 24 hrs. In the third and final treatment the insects were exposed to a filter paper embedded with the same amount of heptyl formate as the rods. Mortality was determined every 24 hrs after which the five rods and the treated filter papers were removed to new jars with new insects. The process was repeated over a 9 day period.

Data Analysis

In those cases where control mortality was observed data was adjusted using the Abbott's formula (Abbott 1925). When control mortality exceeded 10% that rep was discarded. Probit analysis was performed and the LC_{50} and LC_{90} of each insecticide with and without the synergist were estimated (SAS Institute 2003). The data reported in Tables 6-1, and 6-2 include slope, goodness of fit characteristics (chi-square, P-value) and LC_{50} and LC_{90} estimates with 95% confidence limits. LC estimates with non overlapping 95% confidence limits were considered significantly different.

Body-weight corrected LC_{50} of each insecticide for house flies and *Drosophila* were calculated. One hundred individuals from both species were weighed and that weight was recorded. That number was then divided by 10 to give the average weight of 10 house flies and 10 *Drosophila* (0.2126 and 0.006 g, respectively). The average weight of the 10 insects was used to adjust the LC_{50} from mg/liter into mg/ g of insect body weight/liter. The *Drosophila* data were retrieved from Scharf et al. (2006).

PoloPlus 2.0 (2005) was used to calculate the potency ratios of the LC50s with & without the synergist. The program calculated 95% confidence limits for every ratio. The 95% CI were used to determine whether there were significant differences in the LC50s due to the effect of the synergists.

In order to determine heptyl formate release rate form each rod regression analysis was performed (SAS Institute 2003) that showed the relationship between release of heptyl formate vapors and time. The rods were weighed before and after being embedded with heptyl formate. The decrease in the rod weight was recorded through time and the release of heptyl formate was estimated. According to the regression equation [$y=0.0006x-0.0003$ and $R^2= 0.9994$, where y represents heptyl formate weight in grams and x represents time of release in minutes] it would require at least 111.11 hours for 4 g of heptyl formate to be released. Also, SNK (Student-Newman-Keuls) test was performed to determine the day when significant decrease in house fly mortality for the rod (3.81 g) treatment was seen (SAS Institute 2003).

Results

Toxicity Evaluation of Novel Compounds

DDVP was by far the most toxic compound tested on house flies. Specifically, it was 25 times more toxic compared to the second best compound, the heterobicyclic menthofuran. Menthofuran was the most toxic compound among the three compounds tested on house flies

(LC_{50} estimate 3.70 mg/liter). EGDF was the second most toxic compound and heptyl formate was the least toxic compound among the three (LC_{50} estimates 9.27 and 32.62 mg/liter, respectively) (Table 6-1, Table 6-2, Fig. 6-1).

Toxicity Evaluation of Novel Compounds with the Synergistic Effect of DEF and PBO

For heptyl formate and EGDF, when co-applied with DEF, their toxicities decreased significantly. The toxicity of heptyl formate decreased by 1.5 times, whereas the toxicity of EGDF decreased by 2 times. Also, the toxicity of menthofuran increased by 1.5 times, when it was synergized with PBO. All synergist effects were significant at the LC_{50} level.

Effectiveness of Controlled Vapor Release of Heptyl Formate in Killing House Flies

The mortality data among the different treatments are shown in Table 6-3. The control treatment caused no mortality throughout the duration of the experiment, which lasted for 9 days. The filter paper treated with 0.95 g of heptyl formate caused 100% mortality for the first day. The filter paper treated with 3.81 g of heptyl formate caused mortality for days 1, 2, and 3. The rod embedded with 3.81 g of heptyl formate caused mortality throughout the duration of the experiment. Also, it was on the 9^{th} day when significant decrease in house fly mortality was seen.

Discussion

Comparing Toxicities of Novel Compounds Among House Flies and *Drosophila*

This study is the first report of the toxic effects of the novel volatile compounds heptyl formate, EGDF, and menthofuran on house flies. Scharf et al. (2006) reported toxicity of these novel compounds on *D. melanogaster* Meig. They used *Drospophila* as a model to assess potential efficacy of these compounds on mosquitoes and flies. There were significant differences among the toxicities of the compounds on house flies and *Drosophila* (Table 6-2). Overall, all the compounds were more toxic to house flies than *Drosophila*. DDVP was by far the most toxic insecticide for both insects and was significantly more toxic to house flies than

Drosophila. Specifically, DDVP was 5.2 times more toxic to *Drosophila* than house flies. DDVP has been known as a very effective insecticide against various insects for many years. Ihnidris and Sullivan (1956) gave one of the earliest reports regarding the toxicity of DDVP against house flies. They reported 100 % knock down of house flies after 2 hours exposure to DDVP vapors. On average the compounds were approximately by 10 times more toxic to house flies than *Drosophila*. Menthofuran was the most toxic compound when tested on both insects. However, heptyl formate was more toxic than EGDF to *Drosophila* and less toxic than EGDF to house flies.

There are several possible explanations as to why the compounds performed differently on house flies and Drosophila. A first explanation could be differences on the insect handling techniques during the experimentation. Scharf et al. (2006) used CO_2 to knock down *Drosophila* prior to exposing them on the insecticides, whereas I used ice for knocking down house flies. Another explanation could be species differences on acquiring and metabolizing insecticides. The lethal effects of insecticidal compounds depend upon the amount of insecticide that reaches the target site (site of action). The amount of insecticide that reaches the target site is controlled by certain processes such as penetration through the insect cuticle, diffusion through the insect spiracles, bioactivation/biodegradation within the insect body, travel distance to the site of action and finally excretion just to name a few (Quraishi 1977, Matsumura 1980, Yu 2006). Different insect species can differ greatly in susceptibility to insecticidal compounds due to distinct differences in the physical and physiological processes mentioned above. Insecticides with high vapor pressures, such as the insecticides studied in this paper, show the tendency to enter the insect body through the spiracles (Matsumura 1980). Therefore, the susceptibility of an insect to a vapor toxicant is believed to be correlated with its rate of respiration (Vincent & Lindgren

1965). In general, different insects exhibit different patterns of gas exchange when at rest (Lighton 1988, 1990, Lighton & Berrigan 1995). The most familiar and well studied pattern is the Discontinuous Gas Exchange Cycle (DGC) (Kestler 1985, Lighton 1994), where the spiracles remain close for lengthy periods of time allowing for no gas exchange. This close-spiracle phase is followed by a fluttering-spiracle phase and finally an open-spiracle phase where the accumulated CO_2 escapes form the tracheal system to the surrounding environment. Little information, however, is available on the respiratory pattern of small insects (body weight ~ 1 mg) such as *Drosophila* (Williams et al.1997, Williams and Bradley 1998, Lehman et al. 2000, Fielden et al. 2001). What is known so far is that *Drosophila* has the ability to control gas release from its tracheal system. There is some evidence to support that *Drosophila* performs DGC, however further research is needed for more legitimate results. Not much research has been done on the respiratory pattern of houseflies. Due to the absence of evidence one should consider that house flies and *Drosophila* may follow a different breathing pattern. If this is true, the insects may be allowing different amounts of insecticide to enter their body and that may be one of the factors responsible for the different responses they show to the various insecticides tested.

Another explanation could be differences in the detoxification systems among house flies and *Drosophila*. Once the insecticide enters the insect body it is perceived as a foreign substance or xenobiotic, and is metabolized by different metabolical processes with the ultimate goal to be converted into a less toxic polar substance that will eventually be removed form the body. This metabolic process is called “detoxification”. By far the two most significant reactions involving the metabolism of insecticides are the NADPH-requiring general oxidation system and the hydrolysis of esters (Matsumura 1980). The activity of these two enzymatic systems varies

among different insect species, resulting in species differences in susceptibility to various insecticidal compounds (Casida et al. 1976. Brooks 1986, Valles et al. 1997, Scharf et al. 2000).

Implications of the Synergistic Effects of PBO and DEF on the Toxicity of the Novel Compounds on House Flies

The modes of action of menthofuran, EGDF, and heptyl formate on house flies so far remain undefined. According to the work presented in this paper there seems to be a significant effect of cytochrome P450 enzymes on the metabolism of menthofuran. When cytochrome P450 enzymes were inhibited by the action of PBO the toxicity of menthofuran increased by 1.5 times. This finding is in agreement with Nguyen et al. (2007), who showed that P450 plays an important role in methofuran detoxification. They, also, showed evidence supporting P450-based activation of menthofuran.

Also, there was evidence supporting esterase-based activation of both heptyl formate and EGDF. When heptyl formate and EGDF were co-applied with the esterase inhibitor DEF their toxicity decreased by 1.5 and 2 times, respectively. The second finding comes in agreement with both Haritos & Dojchinov (2003) and Nguyen et al. (2007), who supported esterase-based activation of some formate esters. In order for more legitimate conclusions to be made more extensive and complete research needs to be conducted where all of the compounds will be tested in combination with both synergists on susceptible and even resistant fly species.

Structure-activity Relationships

Among the two formate esters EGDF and heptyl formate, the first is significantly more toxic to houseflies than the second by 3.5 times. When looking at their chemical structures there is one difference that stands out; EGDF is composed by two molecules of formic acid, where as heptyl formate is composed by only one. According to Haritos & Dojchinov (2003) findings on alkyl ester mode of action, formate esters were more toxic than other alkyl esters. That was

partially due to their hydrolysis to formic acid. Based on their findings one might say that EGDF was more toxic than heptyl formate because it contains two molecules of formic acid in its structure, and therefore the esterases would release 2 molecules of formic acid when hydrolysing EGDF, as opposed to 1 molecule of formic acid when hydrolyzing heptyl formate.

Controlled Vapor Release of Heptyl Formate

Controlled vapor release of heptyl formate can provide effective house fly mortality over time. In the future these compounds should be embedded in specialized plastic polymers, similar to the DDVP resin strips, that would provide prolonged release of vapors and, therefore, result in prolonged insect mortality.

In conclusion, DDVP was by far the most toxic insecticide for both insects and was significantly more toxic to house flies than *Drosophila*. All three novel compounds were significantly more toxic to house flies than *Drosophila*. Menthofuran was the most toxic compound among the three tested on house flies (LC_{50} estimate 3.70 mg/liter). EGDF was the second most toxic compound and heptyl formate was the least toxic compound.

Table 6-1. Vapor toxicity of EGDF, heptyl formate, and menthofuran with and without the synergistic effect of DEF and PBO and the organophosphate DDVP to house flies *Musca domestica* (L.)

Insecticide	Slope ± SE	LC_{50} mg/liter (95%CI)	LC_{90} mg/liter (95%CI)	χ^2	P	Potency Ratio [b]
DDVP[a]	9.6 ± 1	0.148 (0.14-0.15)	0.202 (0.19-0.22)	2.35	0.67	-
EGDF	7.4 ± 0.8	9.27 (8.75-9.75)	13.81 (12.81-15.36)	9.76	0.14	
EGDF + DEF	7.9 ± 0.67	18.56 (17.76-19.33)	26.88 (25.34-29.02)	8.10	0.23	2 (1.87-2.14)
Heptyl formate	4.1 ± 0.6	32.62 (30.21-35.44)	66.89 (55.88-91.58)	3.87	0.79	
Heptyl formate + DEF	6.9 ± 1.19	48.70 (46.20-51.63)	74.45 (65.94-94.29)	3.76	0.29	1.5 (1.36-1.64)
Menthofuran	10.6 ± 1.13	3.70 (3.58-3.83)	4.88 (4.61-5.32)	4.29	0.12	
Menthofuran + PBO	4.8 ± 1.22	2.43 (1.85-2.69)	4.49 (3.90-6.59)	0.49	0.48	0.65 (0.56-0.76)

[a] Positive control
[b] LC_{50}+synergist / LC_{50}

Table 6-2. Body-weight corrected vapor toxicities of EGDF, heptyl formate, menthofuran and the organophosphate DDVP to house flies *Musca domestica* (L.) and *Drosophila melanogaster* Meig.

Treatment	Housefly LC_{50} (mg/g of insect/liter)	Drosophila LC_{50} (mg/g of insect/liter)[b]
DDVP[a]	0.7 (0.67-0.72)	3.7 (3.2-4.3)
EGDF	44 (41.15-45.86)	834 (676.6-1,846)
Heptyl formate	153 (142.1-166.7)	450 (420-475)
Menthofuran	17.4 (16.83-18)	136 (120-150)

[a] Positive control
[b] *Drosophila* data Scharf et al. 2006

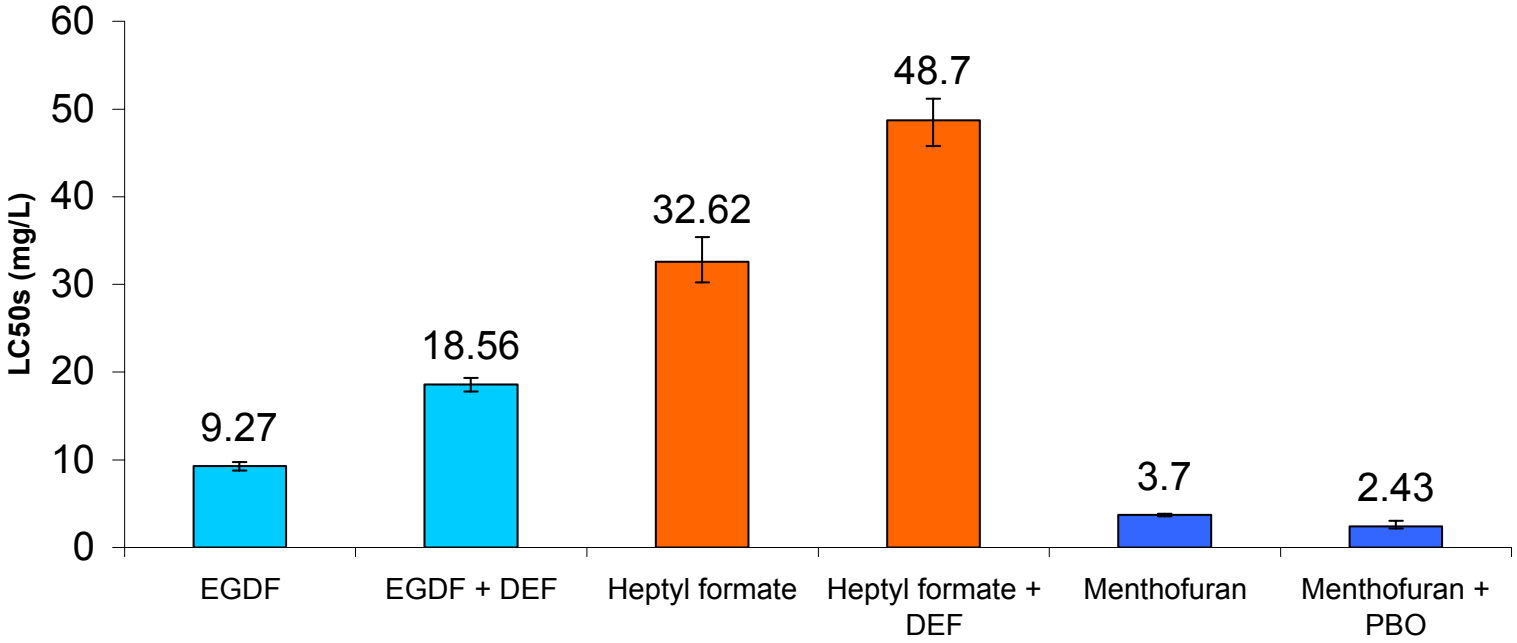

Figure 6-1. Vapor toxicity of EGDF, heptyl formate, and menthofuran with and without the synergistic effect of DEF and PBO to the house flies *Musca domestica* (L.).

Table 6-3. Percent mortality of controlled vapor release of heptyl formate on house flies Musca domestica (L.) over 9 days among 3 different treatments and a blank control

Time (days)	Percent Mortality of Heptyl Formate on House Flies			
	Control	Filter Paper (3.81g)	Filter Paper (0.95 g)	Ceramic Rod (3.81 g)
Day 1	0	100	100	100a
Day 2	0	100	0	100a
Day 3	0	100	0	98 ± 2a
Day 4	0	0	0	96 ± 4a
Day 5	0	0	0	88 ± 5.8ab
Day 6	0	0	0	84 ± 6.8ab
Day 7	0	0	0	82 ± 5.8ab
Day 8	0	0	0	74 ± 10.3ab
Day 9	0	0	0	64 ± 14.7b

Percentages followed by the same letter are not significantly different (SNK test, SAS Institute, 2003)

CHAPTER 7
SUMMARY

The main objective of my research was to evaluate vapor toxicity of novel, low molecular weight compounds with insecticidal activities on mosquitoes and house flies. The results of the experiments have shown that all compounds demonstrated vapor toxicity to both mosquitoes and house flies. However, the organophosphate DDVP was by far the most toxic compound to both mosquitoes and house flies.

A total of 16 insecticidal compounds were tested on mosquitoes: 15 novel compounds (7 formates, 4 acetates, and 4 heterobicyclics) and the organophosphate DDVP. DDVP was 54.4 times more toxic compared to the second best compound, the formate ester methyl formate. Within the novel compounds, overall, formate esters were the most toxic family followed by the heterobicyclics and, last, by the acetate esters. Within the formate group, methyl formate was the most toxic ester (LC_{50} estimate 1.36 mg/liter), followed by butyl, propyl, ethyl, hexyl formate, EGDF, and heptyl formate. The toxicities of propyl and ethyl formate were not significantly different and there was no major significance between them and butyl formate. EGDF and heptyl formate (LC_{50} estimates 2.99 and 3.17 mg/liter, respectively) were the least toxic formate esters with toxicities in the same range as the heterobicyclics, the second best performing family of compounds. Within the heterobicyclic group, coumaran was most toxic (LC_{50} estimate 2.03 mg/liter), followed by benzothiophene, dimethyl-coumarone and menthofuran. Benzothiophene and dimethyl-coumarone were not significantly different. Within the acetate group, hexyl acetate was the least toxic compound (LC_{50} estimate 5.09 mg/liter). The toxicities of propyl, butyl and pentyl acetate were not significantly different.

A total of 4 insecticidal compounds were tested on house flies: two formates, one heterobicyclic, and the organophosphate DDVP. DDVP was 25 times more toxic compared to

the second best compound, the heterobicyclic menthofuran (LC_{50} estimates 3.70). Menthofuran was followed by the formate esters EGDF and heptyl formate (LC_{50} estimates 9.27 and 32.62 mg/liter, respectively).

DDVP has been characterized by EPA as a "probable human carcinogen" and because of its implications in human health its use in 2006 was restricted to confined spaces such as wardrobes, cupboards and closets where no human activity takes place (EPA 2006). Even though the novel compounds did not demonstrate the high vapor toxicity demonstrated by DDVP, they showed good potential to be used as alternative vapor toxicants against mosquitoes and house flies for those situations where the use of DDVP is banned. Their low mammalian toxicities in combination with their pleasant, fruity odors make them very good DDVP replacement candidates. Also, the potential of the novel compounds as contact toxicants should be investigated in the future as they might exhibit good toxicities as contact insecticides, and thus provide an additional tool for the control of public health pests such as mosquitoes and house flies.

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BIOGRAPHICAL SKETCH

Alexandra Chaskopoulou was born on June 3, 1981 in Thessaloniki, Greece, to Efthimios and Kalliopi Chaskopoulos. She has one sister and one brother. She and her family have spent most of their lives in Greece. Upon completion of her high school education in Greece, she decided to come to the United States in order to pursue her college education as an entomologist.

She arrived at the United States in 2003, and within a year she earned her minor in biology from St. Andrews University of Michigan. In 2004 she moved in Gainesville, Florida where she earned her Bachelor of Science degree in entomology from the University of Florida and graduated in 2005. She remained at the University of Florida since 2007, when she completed her master's degree in medical and veterinary entomology.