THE EFFECTS OF AZADIRACHTIN, METHOXYFENOZIDE, AND TEBUFENOZIDE ON THE YELLOW FEVER MOSQUITO, *AEDES AEGYPTI*

by

DANIEL JACK USRY

(Under the Direction of Mark R. Brown)

ABSTRACT

Mosquito control is important to control the spread of pathogens which are transmitted by the bites of infected mosquitoes. To this end, research has focused on insecticides that are insect specific and have relatively low impact on the environment. Here I report on the plant botanical azadirachtin and the ecdysone agonists methoxyfenozide and tebufenozide. Overall, my results suggest that azadirachtin and tebufenozide are not suitable for use as insecticides, even though they cause mortality at high doses. Methoxyfenozide kills mosquitoes at high doses and reduces the number of eggs oviposited by females. Additionally, methoxyfenozide is able to stimulate egg production in non-bloodfed females. However, it is not able to cause eggs to develop all the way to oviposition.

INDEX WORDS: *Aedes aegypti*, Azadirachtin, Methoxyfenozide, Tebufenozide

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DEDICATION

I dedicate this to my family.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The yellow fever mosquito, *Aedes aegypti*, (Diptera, Nematocera, Culicidae) is an anthropogenic species that originated in Africa, but has spread to nearly all tropical and subtropical regions (Jansen and Beebe 2010, Adelman et al., 2002). Originally adapted to ovipositing in stagnant pools, the close association of this species with humans has led to it also ovipositing into a variety of other containers such as water tanks, buckets, pots and used tires (Jansen and Beebe 2010). Eggs oviposited in these containers may lie dormant, up to a year, until rainfall replenishes the pool, and provides an aquatic environment for the eggs to hatch (Jansen and Beebe 2010).

Humans are the preferred hosts of female *Ae. aegypti* for blood meals, and adults often rest in human habitations (Jansen and Beebe 2010). Blood feeding provides amino acids and other nutrients that are required for egg production. Females are considered a nuisance because of the painful bites inflicted during blood feeding and are serious economic pests because of their ability to vector pathogens. Key pathogens transmitted by *Ae. aegypti* include the viruses that cause yellow, dengue, and Chikungunya fever and the filarial worms that cause lymphatic filariasis (Adelman et al., 2002). The transmission cycle begins with a mosquito taking a blood meal from an infected human (or animal, in the case of zoonotic disease). This is followed by a period of extrinsic incubation, which refers to the time between the entrance of the pathogen into the vector, and the time when the vector can transmit the pathogen to another individual. After ovipositing the first batch of eggs, the female must then attempt another reproductive cycle and

take a blood meal, during which infective stages of a pathogen are released into a new host. Bed nets are often used to protect humans from night biting mosquitoes, but these are ineffective against *Ae. aegypti* because it is a day-biting species (Jansen and Beebe 2010). *Ae. aegypti* females often also take small blood meals from multiple hosts, which can further promote disease transmission (Jansen and Beebe 2010).

According to World Health Organization (WHO) estimates, 200,000 reported cases and 30,000 deaths are attributed to yellow fever annually (Tolle et al., 2009). These numbers are confirmed cases, and it is assumed that there are significantly more that go unreported each year. The yellow fever vaccine 17D is widely dispensed in Africa during epidemics, but young children are still susceptible to yellow fever, because they were too young during previous outbreaks to have been immunized (Tolle et al., 2009). Efforts to eradicate yellow fever are hindered by asymptomatic carriers, such as lower primates and lemurs, that act as reservoirs (Tolle et al., 2009). If an infected person or animal moves from the jungle into a densely populated urban area, large outbreaks can occur (Tolle et al., 2009).

The WHO estimates that every year there are 50-100 million new infections and 22,000 deaths attributed to dengue fever. Dengue fever is most common in Central and South America, but recently the Centers for Disease Control and Prevention have investigated outbreaks along the Mexico-Texas border and in Key West, Florida (Radke et al., 2012). An effective vaccine for dengue fever cannot be developed, because there are four distinct serotypes of the dengue virus. Infection with any one of these four serotypes alone will cause dengue fever. A successful vaccine would have to protect against all of these serotypes (Tolle et al., 2009). Dengue fever is also difficult to eradicate because there are reservoir populations of lower primates (Tolle et al., 2009).

Current control methods focus on the interruption of disease transmission by removing the mosquito vectors of disease, thus breaking transmission between humans. Removing or controlling the mosquito vectors lowers or eliminates exposure to new hosts. This is primarily achieved through insecticide treatment to water where mosquito larvae are likely to be present or fogging adults. Recently, scientists have begun trying to find ways to disrupt the processes associated with mosquito reproduction, which is the reason mosquitoes take blood meals and can act as vectors.

Egg maturation in anautogeny

Vitellogenesis is the process by which female insects synthesize and secrete yolk proteins, which subsequently accumulate in the developing oocytes (Attardo et al., 2005). In *Ae. aegypti* and other mosquito species, vitellogenesis is divided into three stages: pre-vitellogenesis, vitellogenesis and post-vitellogenesis (Raikhel et al., 2002).

Pre-vitellogenesis begins soon after the female ecloses from the pupa. After eclosion, the corpora allata releases juvenile hormone (JH), which reaches a peak titer at 48 h post eclosion (Raikhel et al., 2002). This surge in JH prepares the fat body for mobilization of energy reserves stored during the larval stage: nucleoli enlarge three fold, RNA synthesis rates increase, poly(A)-RNA accumulation occurs, ribosomes and rough endoplasmic reticulum proliferate, and Golgi complexes and the plasma membrane extensively invaginate (Attardo et al., 2005, Raikhel et al., 1983, 1990). These changes prepare the fat body to make large amounts of the yolk protein, vitellogenin (Raikhel et al., 1983). The fat body thereafter remains quiescent until a blood meal is taken from a vertebrate host, which triggers the vitellogenic phase. (Attardo et al., 2005).

The vitellogenic phase lasts for 30 – 36 hours, and is characterized by the blood meal stimulating the release of the neurohormones, specifically ovary ecdysteroidogenic hormone (OEH), and insulin like peptides (ILPs) from the medial neurosecretory cells in the brain (Brown et al., 1998, 2008). The actual cause of neurohormone secretion is unknown, although it may occur in response to signals from stretch receptors in the midgut (Klowden et al., 1987). Along with OEH, five of the eight ILP genes encoded by *Ae. aegypti* (ILPs 1, 3, 4, 7, and 8) are expressed in the brain (Wen et al. 2010, Riehle et al. 2006). OEH and the ILPs are secreted into the hemolymph and travel to the ovaries, where they stimulate the production of the steroid hormone ecdysone (Hagedorn et al., 1975, Gulia-Nuss et al., 2011). The ovaries then secrete ecdysone into the hemolymph, where ecdysone is hydroxylated by the fat body into a more active form, 20 hydroxyecdysone (20E), by the cytochrome P450 enzyme, 20-monooxygenase (Attardo et al., 2005).

The ecdysteroid 20E binds to the ecdysone receptor (EcR), plus a second protein named ultraspiracle (USP) (Roy et al., 2007). Binding of 20E to this heterodimer forms the EcR complex, which then binds ecdysone response elements in the promoter region of many genes of particular importance binding of the EcR complex to the promoter region of the vitellogenin gene induces its transcription in the fat body (Hansen et al., 2004). Vitellogenin mRNA is then translated by ribosomes in the rough endoplasmic reticulum, and the vitellogenin protein is processed in the Golgi complex and secreted into the hemolymph. Ecdysteroid titers (specifically 20E) typically peak around one day after the ingestion of a blood meal and slowly decline back to basal levels by 36 h after the blood meal (Attardo et al., 2005). This decrease in 20E is what ultimately causes the end of yolk protein synthesis (Attardo et al., 2005).

In addition to triggering ecdysteroid synthesis, the release of neuropeptides and ILPs stimulates the transcription of late phase trypsin-like enzymes in the midgut, which digest proteins in the blood meal. Resulting amino acids are thereafter absorbed by midgut cells and released into the hemolymph. Amino acid sensing by the fat body further synergizes yolk protein biosynthesis (Roy et al., 2007, Gulia-Nuss et al., 2011).

Understanding that all of these reproductive processes are under precise hormonal control shows us that there are many potential targets for insecticides that could disrupt reproduction and lower pest populations of mosquitoes. This allows for directed research towards the discovery of new and effective alternatives to the synthetic organic insecticides that have been historically used.

Plant botanicals

One source of alternative insecticides is plant botanicals. Interest in botanical insecticides first began in the early 1900s and continued into the 1950s, but waned with development of synthetic organic insecticides (Mulla and Su 1999). In the aftermath of the discovery that these broad spectrum organic insecticides, like DDT, accumulate in the environment and have negative effects on non-target species, there was a push for the development of more environmentally friendly and specific insecticides, again including botanicals.

Some plant botanicals, including pyrethrum, nicotine, azadirachtin, camphor, and turpentine, predate the use of synthetic insecticides (Shaalan et al., 2005). These and other plant botanicals typically are not used in a concentrated liquid form, but in whole plant or powdered form. Examples include powdered chrysanthemum, which contains pyrethrum, or the use of twigs and leaves of the neem tree, which contain azadirachtin (Shaalan et al., 2005).

Plant botanicals were recognized as a useful tool in mosquito control when it was observed that mosquito breeding sites with aged litters of specific plants consistently contained fewer mosquito larvae than sites without the leaf litter (Shalaan et al., 2005). While plant botanicals are effective in controlling the larval stages of mosquitoes, bioactivity against mosquitoes varies widely dependent on the plant species, part of the plant, age of the plant, solvent used to extract the compound, and mosquito species (Shalaan et al., 2005). *Aedes* larvae are heartier and are less susceptible to botanical extracts than *Anopheles* mosquitoes and because of this *Aedes* species are frequently used in screening trials for insecticides (Shaalan et al., 2005).

Azadirachtin

A plant botanical that has received significant attention in recent years is azadirachtin, from the neem tree, *Azadirachta indica*. The neem tree is in the family Meliaceae and has a native range that extends from the Indian subcontinent to the Middle East (Mordue-Luntz et al., 2005). Historically, *Az. indica* was used by the people of the Indian subcontinent in ayurvedic medicine and pest control. Twigs and branches of the tree added to granaries prevented infestations from insects (Mordue-Luntz et al., 2005). The compounds responsible for these repellant effects were first characterized in Sudan in 1966. Heinrich Schmutterer, who was funded by The Anti-Locust Research Center in London (Haskell et al., 1969), noted that when the desert locust, *Schistocerca gregaria*, swarmed in an area and consumed most plants, the locusts would not consume foliage of *Az. indica.* Subsequently, Butterworth and Morgan isolated an active compound, which was a tetranortriterpenoid limonoid, and named it azadirachtin (Butterworth et al., 1968, 1971).

Azadirachtin is only one of many compounds from the neem tree that possess insecticidal properties (Mordue-Luntz et al., 2005). These compounds are not limited to one part of the plant. Fruit, seeds, and bark from the neem tree have all been used successfully to control mosquitoes in lab settings (Howard et al., 2009, Wandscheer et al., 2004). In the event of resistance to azadirachtin alone, different mixtures can be made with the related compounds (Mordue-Luntz et al., 2005).

Azadirachtin has diverse activities. When applied to plants, azadirachtin often acts as an antifeedant against insect herbivores. Insects are deterred because azadirachtin stimulates deterrent receptors in the insect, inhibits phagostimulant receptors, or by a combination of the two (Mordue-Luntz et al., 2005, Linton et al., 1997, Huang et al., 2004). Antifeedant effects range from largely ineffective (as for hematophagous insects, such as *Culex* adults) to incredibly effective (*S. gregaria*), to the point where the insect would rather starve to death than to consume plant material treated with azadirachtin (Linton et al., 1997, Mordue-Luntz et al., 2005). If the insect does eat the material treated with azadirachtin, there is a reduction in feeding, efficiency of protein digestion, and loss of muscle control in the gut (Linton et al., 1997). Treatment of juvenile insects with azadirachtin typically causes delays or inhibits molting, or disturbs the actual process of molting by disrupting ecdysis (Linton et al., 1997) Treatment of adults often delays or disrupts ovarian development (Subrahmanyam et al., 1989).

Based on current information, azadirachtin has more than one mode of action. Initially, it was believed that azadirachtin in some way disrupted ecdysone related functions. Researchers hypothesized that azadirachtin prevented the release of ecdysteroids from prothoracic glands. This was not substantiated because prothoracic glands or brain-ring gland complexes of the blue bottlefly, *Calliphora vicina*, the silkworm, *Bombyx mori*, and the tobacco budworm, *Heliothis*

virescens all produce ecdysteroids in vitro in the presence of azadirachtin plus the neuropeptide, prothoracicotropic hormone (PTTH), which stimulates this process from neurosecretory cells in the brain of insects (Bidmon et al., 1987, Barnby et al., 1990). Koolman et al. (1988) then looked to see if azadirachtin binds to the EcR, which would classify azadirachtin as an agonist. Other experiments showed that azadirachtin exhibited no EcR binding activity (Koolman et al., 1988).

Later studies though showed azadirachtin inhibits the release of neurohormones. Decapitation and ligation experiments with *B. mori*, the triatomid bug, *Rhodnius prolixus*, and the variegated cutworm, *Peridroma saucia*, revealed that PTTH production was inhibited when the study insects were treated with azadirachtin (Mordue-Luntz et al., 2005). Similarly, studies on the green bottle fly, *Lucinia cuprina*, showed that when treated with azadirachtin, there were degenerative effects seen in the nuclei of all endocrine glands, such as the corpora cardiaca, the corpora allata, and the ring gland complex (Meurant et al., 1994). Studies in the migratory locust, *Locusta migratoria*, have shown that azadirachtin prevents the release of neuroendocrine factors from the corpora cardiaca (Subrahmanyam et al., 1989). This blockage is what culminates in the delayed or non-existent ecdysteroid peak in azadirachtin treated immature insects (Mordue-Luntz et al., 2005). Additionally, azadirachtin disrupts cell division, blocks the release and transport of neurosecretory peptides, inhibits spermatozoa formation and interferes with 20-monooxygenase activity (Mordue-Luntz et al., 2005, Smith et al., 1988).

The successful use of azadirachtin in controlling medically important pests has been seen across many orders, including Hemiptera, Diptera, Blattaria, Siphonaptera, Coleoptera, and it even has a negative effect on the trypanosome, *Trypanosoma cruzi*, living inside of *Rhodnius prolixus* (Mulla and Su 1999). Azadirachtin has also been shown in one case to have a negative impact on the immune system of *R. prolixus*. When researchers diluted azadirachtin into

a bloodmeal and fed it to *R. prolixus* adults and then injected them with the bacteria *Enterobacter cloacae* or *Escherichia coli,* they found that there was a significant reduction in hemocyte abundance and nodule formation, antibacterial activity, and wound healing (Azambuja et al., 1991).

Insect Growth Regulators

Insect growth regulators (IGRs) are another alternative group of synthetic or natural compounds that interfere with the physiological growth and development of insects (Ruscoe et al., 1972, Staal et al., 1982). Their insect-specific toxicity usually makes them harmless to nontarget species, like fish, birds and amphibians (Retnakaran et al., 1985). Some IGRs exhibit high acute toxicity on insects at high concentrations, but most cause developmental abnormalities at lower concentrations that ultimately result in death (Ruscoe et al., 1972). Some of the earliest research with an IGR was done by Dr. Carrol Williams, Harvard University, who suggested that juvenile hormone (JH) could be used as an insecticide (Staal et al., 1975).

Scientists initially reasoned that insects would not develop resistance to IGRs because most were analogues of hormones produced naturally by the insect, but some studies document that a few insects have evolved resistance to select IGRs in wild populations (Graf et al., 1993). The most cited drawback of using IGRs as insecticides is that they act more slowly than conventional insecticides, thus resulting in more damage to crop plants (Graf et al., 1993). On the other hand, many IGRs have antifeedant properties which help lower crop damage on plants (Dhadialla et al., 2005). Occasionally, the delayed nature of IGRs is also beneficial, such as when used to treat for termites or ants, where delayed mortality allows a compound to spread throughout the colony (Dhadialla et al., 2005).

IGRs can be subdivided into three categories: 1) ecdysteroid agonists, 2) JH analogues, and 3) chitin synthesis inhibitors (Dhadialla et al., 2005). Ecdysteroid agonists mimic the action of ecdysone and 20E, while JH analogues mimic the action of JH and chitin synthesis inhibitors disrupt chitin formation during molting. The JH mimic, methoprene, works well against the larval stages of mosquitoes and is commercially used under the trade name Altosid (Gordon and Burford 1983). It is commonly added to cisterns and wells and is nontoxic to humans. Other studies have shown that a few ecdysone agonists, such as methoxyfenozide and tebufenozide, have similar effects on mosquito larvae (Beckage et al., 2004).

Tebufenozide and Methoxyfenozide

Tebufenozide (RH-5992) and methoxyfenozide (RH-2485) are substituted analogs of the first generation ecdysteroid agonist, RH-5849, and are both commercially available and marketed by Dow AgroSciences LLC as MIMIC (tebufenozide) and INTREPID (methoxyfenozide) for control of lepidopteran crop pests (Dhadialla et al., 2005). Methoxyfenozide has a broader range of pest control and is more potent than tebufenozide (Dhadialla et al., 2005). Methoxyfenozide and tebufenozide are true ecdysone agonists, because they bind to the EcR (Sundaram et al., 1998).

During molting in insects, some of the associated genes are expressed when the hemolymph ecdysteroid titer rises and are not expressed when the titer is low, while others are repressed when the titer is high but expressed at a low titer. When methoxyfenozide or tebufenozide binds to the EcR, expression of several genes induced by 20E are affected (Dhadialla et al., 2005). For example, high 20E titers repress translation of mRNA for the 14 kDa larval cuticular protein (LCP-14) and do not allow expression of DOPA decarboxylase mRNA in

the midge, *Chironomus tentans* (Dhadialla et al., 2005). Similarly, persistent exposure to methoxyfenozide and tebufenozide also down regulate these transcripts (Dhadialla et al., 2005). Methoxyfenozide or tebufenozide are also not easily metabolized by insects and as such remain bound to the EcR complex and persist longer than a normal 20E peak, resulting in genes that are typically activated by a decreasing 20E titer to not be activated (Dhadialla et al., 2005). This affects adults as well as larvae. *B. mori* pupae injected with five µg of tebufenozide were found to initiate vitellogenesis normally, which is associated with a 20E peak, but were then unable to complete late vitellogenesis and choriogenesis, owing to the fact that these processes are initiated by a peak in 20E, followed by a drop in 20E (Swevers et al., 1999). This suggests that tebufenozide bound to the ecdysteroid receptor complex of *B. mori* and then was not displaced or metabolized.

Methoxyfenozide and tebufenozide are often touted as being Lepidoptera specific, which is not accurate. While methoxyfenozide and tebufenozide do typically have greater effects on lepidopterans, they also show effects on dipterans and coleopterans (Dhadialla et al., 2005). This is attributed to methoxyfenozide and tebufenozide binding with greater affinity to the lepidopteran EcR than to that of Coleoptera or Diptera (Sundaram et al., 1998). The difference in binding affinity is significant, with one study showing that tebufenozide bound to the EcR of the Indian meal moth, *Plodia interpunctella,* with a 64-fold higher affinity than it bound to the *Drosophila* ecdysteroid receptor complex, suggesting that it takes higher concentrations to have the same effects on dipterans as on lepidopterans (Sundaram et al., 1998).

Purpose of Research

The preceding literature review indicates that azadirachtin has diverse insecticidal activities, while IGRs like methoxyfenozide and tebufenozide are effective insecticides against a number of insects. However, most of the studies on the effects of azadirachtin on mosquitoes focus on larvae. There are few studies on the effects of methoxyfenozide and tebufenozide on mosquitoes, with no research on adults. The goal of my thesis, therefore, was to characterize the lethal and sublethal effects of azadirachtin, methoxyfenozide and tebufenozide on adult female *Ae. aegypti*. Chapter two focuses on the effects of azadirachtin on toxicity and egg maturation in *Ae. aegypti*. Chapter three focuses on the effects of the ecdysone agonists methoxyfenozide and tebufenozide, on oogenesis and vitellogenesis and also to determine if methoxyfenozide stimulates vitellogenesis in female mosquitoes.

CHAPTER 2

THE EFFECTS OF AZADIRACHTIN ON ADULT FEMALE MORTALITY AND EGG MATURATION IN *AEDES AEGYPTI*.

Introduction

Azadirachtin is a plant botanical that has diverse insecticidal activities on a wide range of insects. The effects of azadirachtin on mosquitoes have received some attention, but most of the studies have focused on treating larvae. Azadirachtin has been shown to disrupt molting and cause mortality of larval mosquitoes in the genera *Anopheles, Culex,* and *Aedes* (Su and Mulla 1999). Studies with the Indo-Pakistan malaria mosquito, *Anopheles stephensi,* further show that 0.025 parts per million kills around 70% of all larvae and pupae when added to rearing pans (Nathan et al., 2005). When treated with a lower concentration, 0.01 parts per million, azadirachtin increased the duration of the larval stage by 2 days and pupae eclosed 2 days later than the controls, thus displaying sublethal effects on development (Nathan et al., 2005).

Limited studies suggest that azadirachtin is also insecticidal toward adults. For example, Ludlum et al. (1988) looked at the effects azadirachtin had on *Ae. aegypti* when added to a blood meal. They found that feeding was not inhibited and that ingestion of azadirachtin failed to inhibit or delay oviposition. *In vitro* experiments showed that incubating ovaries with azadirachtin and head extract showed similar levels of ecdysteroid synthesis as ovaries that were incubated with head extract alone (Ludlum et al., 1988). They hypothesized that azadirachtin affected release of neuropeptides from the head, rather than directly effecting downstream effects during oogenesis. Ludlum et al. (1988) also showed that mosquitoes were able to rapidly

metabolize the ingested azadirachtin, with only 5% being recoverable from whole bodies two hours after the blood meal (Ludlum et al., 1988).

When adult *Anopheles stephensi* were exposed to filter paper impregnated with azadirachtin at 0.025 parts per million, approximately 70% adult mortality was seen (Nathan et al., 2005). When treated with 0.01 parts per million, adult female longevity was reduced by 10 days and females laid, on average, 34 fewer eggs per clutch (Nathan et al., 2005). Boschitz similarly found that females were shorter lived and produced fewer eggs when reared, as larvae, in water containing sublethal amounts of azadirachtin (Boschitz et al., 1994). Reduced egg laying due to azadirachtin treatment has also been observed in the southern house mosquito, *Culex quinquefasciatus* (Su and Mulla 1999).

The reduced fecundity of mosquitoes has been hypothesized to be due in part to azadirachtin interfering with 20-monooxygenase activity in the fat body in *Ae. aegypti* (Smith et al., 1988). Ecdysone secreted by the ovaries is converted into 20E by 20-monooxygenase in the fat body. The 20E then activates transcription of genes that encode vitellogenic precursors. With azadirachtin inhibiting 20 hydroxyecdysone production yolk precursor proteins never get produced.

The goal of this study was to determine the effects of azadirachtin on adult mortality and reproduction in Ae. *aegypti* females when applied in ways that mimic current methods, contact (bednet), topical application (aerial spray), and feeding. I then examined whether azadirachtin affected the number of eggs oviposited by the females. Here I report that topical application is the most effective delivery method for killing adult stage *Ae. aegypti*, while feeding of intermediate doses reduced the number of eggs laid per female. Overall, my results suggest azadirachtin is not a useful insecticide for control of adult *Ae. aegypti*.

Materials and Methods

Insects and azadirachtin. The UGAL strain of *Ae. aegypti* was reared as described in Riehle et al. (2006). Adult females were blood fed on anesthetized rats when applicable. Azadirachtin powder (43.82% purity) was obtained from Gowan Company (P.O. Box 5569, Yuma, AZ 85366-5569).

Azadirachtin exposure via impregnated filter paper. A stock solution of 200 µg/µl of azadirachtin was made in ethanol (100%). The stock solution was then added to 500 µl of ethanol to get 5000, 1000, and 500 µg in separate tubes. Filter paper (Whatman 15 cm qualitative) was cut into 2.9 cm X 9.1 cm strips and 4 cm X 4 cm squares. Solutions of 400 µl were pipetted onto the strip and 100 µl were pipetted onto the square. The ethanol was allowed to evaporate. The filter paper strips were then used to line small cylindrical cages.

For experiments with non-blood fed females, ten, three to five day old adult *Ae. aegypti* were placed in each cage, with each cage having one of the filter paper treatments. The mosquitoes were given 5% sucrose water constantly. Mortality was assessed every day for five days. Five replicates were performed.

For experiments with blood fed females, three to five day old, adult *Ae. aegypti* were allowed to feed to repletion on an anaesthetized rat. Ten mosquitoes were placed in each cage one hour after blood feeding, with each cage containing treated filter paper. The mosquitoes were placed in individual cages for egg laying 48 h after blood feeding. Eggs were counted 96 h after blood feeding. Mortality was assessed daily. Sucrose water (5%) was provided constantly. Two replicates were performed.

Azadirachtin topical exposure. The stock solution of 200 µg/µl was serially diluted in 100% ethanol for a range of doses. The treatment solutions were loaded into a 10 µl glass syringe using the Micro 4 MicroSyringe Pump Controller (World Precision Instruments). Mosquitoes were treated with 0.5 µl of a solution dispensed directly on the abdomen.

For experiments with non-blood fed females, ten, three to five day old, adult female *Ae. aegypti* were anesthetized on ice and treated as above. Mosquitoes were then placed ten to a cage and given 5% sucrose water daily. Mortality was assessed daily. At least two replicates were performed.

For experiments with blood fed females, three to five day old, adult *Ae. aegypti* were allowed to feed to repletion on an anaesthetized rat. Mosquitoes were treated as above one hour after blood feeding. Mosquitoes were then placed ten to a cage and given 5% sucrose water daily. Mosquitoes were placed into individual cages for egg laying 48 h after blood feeding. Eggs were counted 96 h after blood feeding. Mortality was assessed daily. Three replicates were performed.

Azadirachtin ingestion. The stock solution of azadirachtin was diluted to 10 µg/µl. This was then further diluted with 5% sucrose water to get concentrations of 0.02, 0.2, and 2 µg/µl in a total of 500 µl. Ten sugar starved, female *Ae. aegypti* were placed into a cylindrical cage. A cotton roll was placed on top of the cage and 500 µl of a treatment was added. The mosquitoes were allowed to feed overnight. Mosquitoes that had not sugar fed were removed from the cages. Mosquitoes were given a blood meal on an anesthetized rat one day post sugar feeding. Individuals that did not take a blood meal were removed. Mosquitoes were placed in individual cages for egg laying 48 h post blood meal. Eggs were counted 96 h post bloodmeal. Mortality was assessed daily. Three replicates were performed.

Results

Azadirachtin contact exposure. I first examined whether azadirachtin had sublethal or lethal effects on non-blood fed and blood fed females when exposed to azadirachtin treated filter paper. Non-blood fed females were observed for five days and no mortality resulted from contact with any of the doses applied to filter paper (Table 2.1). Little or no mortality occurred after a blood meal, but prior to oviposition. Across all treatments, females also oviposited a similar number of eggs (ca.100) (Table 2.2). However, after ovipositing, mosquitoes exposed to 1000 or 5000 µg of azadirachtin exhibited higher mortality than females exposed to 500 µg of azadirachtin or the ethanol control (Table 2.2).

Azadirachtin topical exposure. In contrast to contact application, topical application of azadirachtin to mosquitoes produced a dose-dependent mortality response in which all mosquitoes treated with ≥ 100 µg died within 48 h (Table 2.3).

Topical application of azadirachtin on non-blood fed females resulted in higher mortality at higher doses. After 120 h, doses equal to or greater than 12 µg resulted in over 50% mortality, with doses of 100 µg and 200 µg resulting in 100% mortality. Doses of 6 µg or lower resulted in mortality around 10% (Table 2.3). Mosquitoes that were topically treated 24 h before a blood meal showed low mortality pre-oviposition with a sublethal (<50% mortality) dose range between 0.075 µg and 10 µg of azadirachtin (Table 2.4). The only treatment that exceeded 20% mortality was 10 µg, which resulted in 50% mortality. Mortality post oviposition was above 50% in all treatments, with the exception of 0.075 and 0.3 µg (Table 2.4). The number of eggs oviposited was between 80 and 100 eggs across all doses except the 10 µg dose, which oviposited an average of 51 (Table 2.4).

Mosquitoes topically treated 1 h after a blood meal had less than 10% mortality pre oviposition across all treatments except for the 1 µg dose with 20%. Post oviposition mortality was greater than or equal to 70% across all doses except for the 5 µg dose at 53% (Table 2.5). This is potentially due to cages drying after oviposition rather than due to the effects of azadirachtin itself. The number of eggs females oviposited decreased with increasing doses of azadirachtin. Control females oviposited an average of 92 eggs, while females treated with a 10 µg dose oviposited an average of 48 eggs (Table 2.5)

Azadirachtin ingestion. Feeding azadirachtin to mosquitoes in sugar water only inhibited sugar feeding slightly at the highest dose tested (2 µg), with 5 fewer feeding on the 2 µg dose as compared to the control (Table 2.6). Mosquitoes refused doses higher than 2 µg. Mosquitoes that took a sugar meal containing azadirachtin were not deterred from taking a blood meal 24 h later. Mortality pre oviposition was 0% across all doses, but mortality post oviposition was over 50% across all doses (Table 2.6). Egg counts averaged 73 eggs for the control, 66 for 0.02, and 61 for 0.2 µg, but dropped to an average of 24 eggs at the 2 µg dose (Table 2.6).

Discussion

In the present study, female *Ae. aegypti* that were exposed to azadirachtin on filter paper showed no appreciable mortality, regardless of having been blood fed, or change in number of eggs oviposited. Nathan et al. (2005) used a similar experimental set up with *Anopheles stephensi,* but observed reduced female longevity and fecundity and fewer eggs hatching. This could be attributed to differences between susceptibilities in species. Alternatively, the lack of effects on *Ae. aegypti* could have been due to minimal contact through tarsi and inability of azadirachtin to cross the cuticle into the insect's body. Topical application of azadirachtin in

ethanol to *Ae. aegypti* also had little effect on mortality and number of eggs oviposited, except at the highest doses tested. Ethanol is capable of carrying many compounds across the insect cuticle and is assumed to be able to carry azadirachtin, but this may not be the case. If not, it would explain why no effects were seen from the treatment with azadirachtin.

In the present study, a complete inhibition of egg development was not observed when azadirachtin was ingested. Smith (1988) showed that azadirachtin at 1 mM, incubated with the homogenate of two female abdomens, disrupted 20-monooxygenase (compared to the control, the activity was diminished 80%) activity in the fat body of *Ae. aegypti*. This would suggest that treatment with azadirachtin should decrease the number of eggs seen, because the fat body would not be able to make enough 20E and subsequently vitellogenin to package into the eggs. This is not what I observed. However, significantly fewer eggs were oviposited by females that drank the 2 µg azadirachtin sucrose solution, which is similar to effects seen when newly eclosed female *Culex quinquefasciatus* were fed 10 or 50 ppm azadirachtin (Su et al., 1999). This lack of inhibition of sugar feeding is similar to the findings of Ludlum and Sieber (1988), who noted that *Ae. aegypti* blood feeding was not inhibited when azadirachtin was mixed into a sugar meal, but the finding that 2 µg of azadirachtin causes fewer eggs to oviposited conflicts with Ludlum and Sieber, who saw no reduction in number of eggs oviposited. Ludlum and Sieber noted that very little azadirachtin was recoverable from their mosquitoes after 2 h, possibly being eliminated too quickly to have any effect. One possible explanation for reduction in the number of eggs oviposited in my experiment is that sugar meals are stored in the crop and small amounts are released into the midgut for digestion, which could have acted as a slow release mechanism for the azadirachtin.

Overall, I conclude that azadirachtin is not an effective contact or oral insecticide against adult *Ae. aegypti*. It is possible an azadirachtin product, such as neem oil would be successful, but azadirachtin apparently failed to cross the mosquito cuticle. Azadirachtin could potentially be used as a topical insecticide, but only if used at very high concentrations. In contrast, oral delivery had minimal effects on mortality, but did reduce egg development.

Studies with adult mosquitoes have shown that azadirachtin has deterrent and anti ovipositional effects (Mulla and Su 1999). Reduced oviposition due to azadirachtin treatment has also been observed in *Culex* as well as *Anopheles* species (Su et al., 1999).

Table 2.1: Cumulative mortality of non-blood fed *Ae. aegypti* with continuous exposure to azadirachtin.*

	Ethanol	500 µg	1000 µg	5000 µg
N	50	50	50	50
Mortality at 24 h	0	0	0	0
Mortality at 48 h	0	0	2	0
Mortality at 72 h	1	0	2	0
Mortality at 96 h	1	0	2	0
Mortality at 120 h	1	0	2	0
Total percent mortality	2%	0%	4%	0%

*Data from five replicates, 10 females each

Table 2.2: Cumulative mortality and average number of eggs oviposited by blood fed *Ae. aegypti* with continuous azadirachtin exposure for 48 h.*

	Ethanol	500 µg	1000 µg	5000 µg
N	20	20	20	20
Total dead at 24h	0	1	0	1
Total dead at 48h	0	1	0	1
Total dead at 72h	0	1	1	1
Total dead at 96h	0	1	1	1
Percent Mortality pre-oviposition	0%	5%	5%	5%
Percent Mortality post oviposition	30%	20%	70%	60%
Average egg # (N)	97 (9)	103 (7)	105 (7)	94 (8)

*Data from two replicates, 10 females each

Table 2.3: Cumulative mortality of non-blood fed *Ae. aegypti* by topical application of azadirachtin.*

µg / female	0	0.75	1.5	3	6	12	25	50	100	200
N	50	30	30	30	30	50	30	30	30	30
Total dead at 24h	2	0	0	0	1	11	10	16	25	28
Total dead at 48h	3	0	0	0	1	16	19	22	30	30
Total dead at 72h	5	1	1	0	1	20	23	26	30	30
Total dead at 96h	5	2	3	0	2	24	24	26	30	30
Percent Mortality at 120h	10%	10%	15%	0%	10%	48%	80%	86.6%	100%	100%

*Data from three replicates

Table 2.4: Effects of topical azadirachtin treatment 24 h before a blood meal on pre and post oviposition mortality and number of eggs oviposited in *Ae. aegypti*.

µg / female	0	0.075	0.1	0.3	0.6	1	5	10
N	50	20	50	20	20	50	30	30
Mortality pre-oviposition	6%	10%	20%	0%	15%	18%	17%	50%
Mortality post oviposition	48%	40%	74%	35%	50%	72%	76%	93%
Average egg # (N)	89 (33)	103 (13)	97 (36)	84 (17)	88 (13)	80 (32)	89 (12)	51 (15)

Table 2.5: Percent mortality and number of eggs oviposited after being treated topically with azadirachtin 1 h after a blood meal. *

µg / female	0	0.1	1	5	10
N	30	30	30	30	30
Mortality pre-oviposition (48 h post blood meal)	5%	7%	20%	7%	7%
Mortality post oviposition (96 h post blood meal)	73%	70%	73%	53%	87`%
Average egg # (N)	92 (28)	82 (26)	86 (24)	74 (26)	48 (23)

*****Data from three replicates, 10 individuals each

Table 2.6: Willingness to blood feed, mortality, and number of eggs oviposited by *Ae. aegypti* that ingested azadirachtin in a sugar water.

µg/µl	0	0.02	0.2	2
N	32	32	32	32
Number that sugar fed	31	29	27	26
Number that blood fed	31	29	27	25
Mortality pre-oviposition (48 h post blood meal)	0%	0%	0%	0%
Mortality post oviposition (96 h post blood meal)	52%	66%	67%	88%
Average egg # (N)	73 (24)	66 (21)	61 (18)	24 (18)

*Data from three replicates

CHAPTER 3

THE EFFECTS OF METHOXYFENOZIDE AND TEBUFENOZIDE ON MORTALITY AND EGG MATURATION IN ADULT, FEMALE *AEDES AEGYPTI.*

Introduction

Methoxyfenozide and tebufenozide are IGRs and ecdysone agonists that have insecticidal activity on lepidopterans. The effects of methoxyfenozide and tebufenozide on mosquitoes have received very little attention, with only one study focusing on larval stage of *Ae. aegypti, Culex quinquefasciatus* and *Anopheles gambiae* (Beckage et al., 2004). The authors found that 0.4 µg/µl of methoxyfenozide and ~1.5 µg/µl of tebufenozide in water resulted in 90% larval mortality for each species (Beckage et al., 2004). The cause of death is similar to that of lepidopteran larvae treated with methoxyfenozide or tebufenozide. A new cuticle was produced and separated from the old cuticle by an ecdysial space, but the larvae were unable to shed the old head capsule and eclose from the old cuticle (Beckage et al., 2004). The effects of methoxyfenozide or tebufenozide on adult mosquitoes have not been reported.

As ecdysteroid agonists, methoxyfenozide and tebufenozide should not only be toxic to mosquitoes, but should also have sublethal effects, given the critical role 20E plays in regulating egg maturation. Studies have shown that injections of ecdysone or 20E stimulate vitellogenesis in non-blood fed female *Ae. aegypti* (Fallon et al. 1974, Spielman et al. 1971). Lea (1981) looked at the effects of 20E and ecdysone over a wide range of doses in sugar fed females and found that follicular growth was stimulated, but protein and lipid content of oocytes was lower than controls.

The goal of this study was to determine the effects of methoxyfenozide and tebufenozide on adult mortality and female reproduction in *Ae. aegypti* by contact and topical application. My results indicated that both compounds were toxic at high levels, but tebufenozide had no other effects, while methoxyfenozide reduced the number of eggs oviposited. Methoxyfenozide also, at sublethal doses, stimulated yolk deposition in decapitated blood fed females and intact non-blood fed females.

Materials and Methods

Insects and compounds. Granular methoxyfenozide (99.9% purity) and tebufenozide (99.1% purity) were obtained from Dow AgroSciences LLC, Indianapolis, IN 46268.

Methoxyfenozide and tebufenozide contact exposure. Stock solutions of 40 µg/µl of methoxyfenozide and tebufenozide were made in ethanol (100%). Stock solution was then added to 500 µl of ethanol to get 5000, 1000, and 500 µg in separate tubes. Filter paper (Whatman 15 cm qualitative) was cut into 2.9 cm X 9.1 cm strips and 4 cm X 4 cm squares. A solution was selected and 400 µl were pipetted onto the strip and 100 µl was pipetted onto the square. The ethanol was allowed to evaporate. The filter paper strips were then used to line small cylindrical cages.

Ten, four day old female *Ae. aegypti* were placed in each cage, with each cage having one of the filter paper treatments. The mosquitoes were given 5% sucrose water constantly. Mortality was assessed every day for five days.

Four day old female *Ae. aegypti* were allowed to feed to repletion on an anaesthetized rat. Ten mosquitoes were placed in each cage, with each cage having one of the filter paper treatments, one h later. The mosquitoes were placed in individual cages for egg laying 48 hours

after blood feeding. Eggs were counted 96 h after blood feeding. Mortality was assessed daily. Sucrose water (5%) was given constantly.

Methoxyfenozide and tebufenozide topical exposure. The stock solution of 40 µg/µl was serially diluted to produce doses of 0.25, 2.5, 5, 10, and 20 µg/µl. The treatment solutions were loaded into a 10 µl glass syringe using an auto injector. Three to five day old, adult female *Ae. aegypti* were anesthetized on ice. Mosquitoes were then treated with 0.5 µl of a solution dispensed directly from the syringe onto the abdomen. Mosquitoes were then placed ten to a cage and given 5% sucrose water daily. Mortality was assessed daily.

Three to five day old female *Ae. aegypti* were allowed to feed to repletion on an anaesthetized rat. Mosquitoes were treated with 0.5 µl of a solution dispensed directly from the syringe onto the abdomen one hour after blood feeding. Mosquitoes were then placed ten to a cage and given 5% sucrose water daily. Mosquitoes were placed into individual cages for egg laying 48 h after blood feeding. Eggs were counted 96 h after blood feeding. Mortality was assessed daily.

Three to five day old female *Ae. aegypti* were treated with 0.5 µl of a solution dispensed directly from the syringe onto the abdomen. Mosquitoes were then placed ten to a cage and given 5% sucrose water daily. Mosquitoes were allowed to feed to repletion on an anesthetized rat 24 h after treatment. Mosquitoes were placed into individual cages for egg laying 48 h after the blood meal. Eggs were counted 96 h after blood feeding. Mortality was assessed daily.

Larval mortality from rearing in methoxyfenozide. A stock solution of 40 µg/ µl methoxyfenozide was diluted serially so that 0, 10, 100 and 1000 µg doses of methoxyfenozide were available in 20 µl of ethanol. 10 µl of Tween-20 was added to the 20 µl of methoxyfenozide. This mixture was added to one ml of water to ensure solubility followed by

dilution to 100 ml with deionized water in a 120 ml specimen cup. 50 newly hatched *Ae. aegypti* larvae were added to the cup. Mosquitoes were reared to adulthood. Sucrose water (5%) was offered two days post eclosion. An egg cup was provided.

Sublethal effects of methoxyfenozide and tebufenozide treatment

1. Stimulation of yolk deposition by methoxyfenozide in decapitated, blood fed females. Three day old female *Aedes aegypti* were given a blood meal and, after one hour, the mosquitoes were decapitated and treated topically with a dose of ethanol, 20E, ecdysone or methoxyfenozide. Ovaries were dissected 24 hours post treatment to measure yolk deposition.

2. Induction of autogeny by methoxyfenozide and tebufenozide in non-blood fed females. Four day old, non-blood fed female *Ae. aegypti* were treated topically with different amounts of methoxyfenozide or tebufenozide.

2.1 Yolk deposition. Ovary dissections were performed on half of the experimental females to measure yolk deposition 24 h after treatment. Ovary dissections were performed on the other half of experimental females to measure yolk deposition 48 hours after treatment.

2.2 Confirmation of vitellogenin within the oocytes of methoxyfenozide or tebufenozide treated females. Abdominal wall/fat body, and ovaries were dissected at different time points in physiological saline and placed in sample buffer containing three parts Roche protease inhibitor cocktail (1X) and one part Laemelli 4X sample buffer. Samples were then run on 4–20% SDS-PAGE gels, transferred to nitrocellulose membrane (Protran 0.2 µm, Whatman), and probed with *Ae. aegypti* Vg (R2,1; 100,000) polyclonal antisera. Primary antibodies were then detected using a peroxidase-conjugated goat anti-rabbit secondary antibody (Sigma) and visualized with chemiluminescent substrate (Amersham ECL), as outlined by Gulia-Nuss et al. (2011).

2.3 Trypsin and Chymotrypsin-like activity. At 24 h and 48 h, abdomen walls and were midguts dissected. Abdomens were frozen in 100 µl of 1 mM HCl and midguts were frozen in 100 µl of Tris buffer. A chymotrypsin assay was performed on the abdomen walls, and a trypsin assay was performed on the midguts.

2.4 Ecdysteroid RIA. Ovaries were collected at 24 h and 48 h for incubation (same as above) and an ecdysteroid RIA was performed.

2.5 Induction of autogeny using topically applied ecdysone or 20E. Anhydrous ecdysone and 20E were solubilized in ethanol to make 5 µg/µl stock solutions. 20E was diluted in ethanol to make doses of 0.05, 0.5, 1, 2.5, and 5 µg/µl. Ecdysone was diluted with ethanol to make 0.25, 0.5, and 1 µg/µl doses. Non-blood fed female *Ae. aegypti* received 1 µl topically to the abdomen at. 20 mosquitoes were treated at each dose and time point.

Ovary dissections were performed at 24 h and 48 h after treatment on 10 individuals each. Ovaries were incubated for six hours and an ecdysteroid radioimmunoassay (RIA) (as outlined in Sieglaff et al., 2005) was performed on the samples. At the same time points, abdomen walls were collected and frozen in 100 µl of 1 mM HCL. To assay chymotrypsin-like activity (Masler et al., 1983). Later, thawed samples were transferred to 200 µl of 0.1 mM cold HCl, homogenized, sonicated and centrifuged. Supernatant was collected and 0.05 tissue/sample equivalent was added to a well with 140 µl of 20 mM Tris (pH 7.0) and 8 µl 20 mM CaCl2, followed by 100 µl benzoyl-L-tyrosine ethyl ester (BTEE) substrate. Samples were incubated for 5 min, loaded in wells of a Greiner UV compatible 96 well plate for measurement of absorbance at 256 nm (Biotek plate reader). Activity was quantified based on α-chymotrypsin standards (bovine pancreas, Sigma C4129).

2.6 Host seeking in females exhibiting autogeny after treatment with methoxyfenozide or an ecdysteroid. Female mosquitoes were collected 24 and 48 h after eclosion. Females were topically treated with 0.5 µl of ethanol, 0.25, 0.5, or 1 µg of ecdysone, 0.05, 0.5, 1, 2.5 or 5 µg of 20 hydroxyecdysone, or 2.5, 5, 10, or 20 µg of methoxyfenozide. At 24 and 48 h post treatment, an anesthetized rat was offered for 15 minutes. Blood feeding was considered successful if over half of the mosquitoes took a blood meal.

Effects of methoxyfenozide and tebufenozide on ecdysteroid production in vitro. For both experiments, 50 µl of saline was added to 0.6 ml polypropylene tube caps along with 100, 500, 1,000, 5,000, or 10,000 pg of methoxyfenozide or tebufenozide solubilized in 10 µl of ethanol. Two pairs of ovaries were placed in each cap and were incubated at 37°C. After six hours, 50 µl of the 60 µl in the cap was pipetted out and frozen at 80°C. An ecdysteroid RIA was then performed on the samples from both experiments. For the first experiment, ovaries were dissected from five day old, non-blood fed females. For the second experiment, ovaries were dissected from five day old, 24 h blood fed, females.

Results

Methoxyfenozide and Tebufenozide contact exposure. I examined the effects of contact exposure on non-blood fed and blood fed females to determine if the compounds in a dry form were toxic or had any effect on egg development.

Non-blood fed females that were exposed to tebufenozide and methoxyfenozide on filter paper were observed for five days and little or no mortality resulted from the treatments (Tables 3.1 and 3.2), with the exception of 5000 µg of tebufenozide, which resulted in 80% mortality by 120 h post treatment.

Females taking a blood meal before exposure to methoxyfenozide or tebufenozide treated filter paper suffered no pre oviposition mortality. Post oviposition mortality was less than 50% for tebufenozide and more than 50% for methoxyfenozide (Tables 3.3 and 3.4). The number of eggs laid by these females averaged around 100/female across a range of doses for both compounds (Tables 3.3 and 3.4)

Methoxyfenozide and tebufenozide topical exposure. In contrast to my contact exposure assays, topical application of methoxyfenozide and tebufenozide dose dependently killed non-blood fed females within 5 days of application. Consistent with earlier studies of Lepidoptera, methoxyfenozide exhibited greater toxicity than tebufenozide. Non-blood fed females that received the highest topical doses of methoxyfenozide or tebufenozide showed the highest mortality. For tebufenozide, at a lower dose, only10 or 20 µg, yielding 70% and 100% mortality respectively after 120 h (Table 3.5). Methoxyfenozide at 120 h yielded 90% mortality at 2.5 µg, 80% at 5 µg, and 100% at 10 and 20 µg at 120 h (Table 3.6).

Females taking a blood meal 24 h before topical treatment with tebufenozide or methoxyfenozide had low mortality pre oviposition. The highest mortality was 30% at the 20 µg dose. Mortality post oviposition was high with every treatment of 5 µg or more resulting in over 50% mortality (Table 3.7, 3.8). There was no difference in number of eggs oviposited in females treated with different doses of tebufenozide (Table 3.7). Females treated with methoxyfenozide oviposited fewer eggs as the dose increased. Oviposition did not occur when females were treated with 20 µg of methoxyfenozide (Table 3.8).

Females taking a blood meal 1 h after treatment with tebufenozide had low mortality pre oviposition. All treatments exceeded 50% mortality post oviposition except for the control and 20 µg treatments (Table 3.9). Mosquitoes treated with methoxyfenozide had mortality greater

than 50% for the 5, 10 and 10 µg pre oviposition doses. Post oviposition, the control had close to 50% mortality and the rest had approximately 90% mortality or higher (Table 3.10). No difference in number of eggs oviposited was observed in tebufenozide treated females (Table 3.9). Females treated with methoxyfenozide oviposited fewer eggs as the dose increased (Table 3.10).

Larval mortality from rearing in methoxyfenozide. I performed this experiment as a confirmation of the larval mortality reported in Beckage et al. (2004). Methoxyfenozide at 100 and 1000 µg per 100 ml killed all 50 larvae after 72 h, but control larvae and ones treated with 10 µg per 100 ml experienced 30% mortality and had successful adult emergence (Table 3.11). Larval mortality was due to a lethal molt, with clearly observed head capsule slippage and the old cuticle being retained.

Sublethal effects of methoxyfenozide and tebufenozide treatment

1. Stimulation of yolk deposition by methoxyfenozide in decapitated, blood fed females. I treated decapitated blood fed females to determine whether or not methoxyfenozide would activate the signaling cascade leading to vitellogenesis without neuroendocrine signals from the head. Tebufenozide was not investigated because of lower efficacy as compared to methoxyfenozide.

Methoxyfenozide over the dose range stimulated yolk deposition of 100 µm in oocytes, as did treatment with 20E 24 h post treatment. Treatment with ecdysone resulted in oocytes containing 10 µm of yolk (Fig 3.1).

2. Induction of autogeny by methoxyfenozide and tebufenozide in non-blood fed females. Given that treatment with methoxyfenozide caused decapitated blood fed females to

deposit yolk into oocytes, I wanted to determine if treatment with methoxyfenozide would stimulate vitellogenesis in non-blood fed females. This experiment would let us determine if methoxyfenozide would cause yolk deposition in the oocytes, if what was going into the oocytes was vitellogenin, and where the nutrients to make this protein were being derived in non-blood fed females.

2.1 Yolk deposition. When non-blood fed females were treated topically with a sublethal range of methoxyfenozide or tebufenozide and examined 24 h later, yolk deposition was observed at all doses except for the control. For females treated with tebufenozide, yolk deposition was less than 50 µm of yolk in the oocytes of females for all treatments at 24 h and always increased 48 h, with the 10 µg dose doubling (Table 3.12). For females treated with methoxyfenozide, all treatments had deposited between 59 and 67 µm of yolk at 24 h and at 48 h all doses approximately doubled their yolk deposition, with the exception of the 0.25 µg dose (Table 3.13). However, oviposition was never observed in these females.

2.2 Confirmation of vitellogenin within the oocytes of methoxyfenozide or tebufenozide treated, non-blood fed, females. This experiment was performed to determine whether or not the yolk seen in the oocytes was vitellogenin. After performing a western blot with abdomens from methoxyfenozide and tebufenozide treated female mosquitoes, the 220 kDa band of vitellogenin was seen, indicating that the fat bodies were producing vitellogenin (Fig 3.2).

2.3 Trypsin and chymotrypsin-like activity. Trypsin like activity could not be detected in dissected midguts of females treated with either compound, but chymotrypsin like activity was detected in the fat body at low levels for the 5, 10, and 20 µg (Fig 3.3) for tebufenozide and 2.5 µg, 5 µg, and 10 µg treatments (Fig 3.4) for methoxyfenozide.

2.4 Ecydsteroid RIA. The ecdysteroid RIA was performed to determine if treatment with methoxyfenozide was causing the mosquito to produce ecdysteroids. At 24 h, fewer than 20 picomols was produced across all doses except for 20 µg. At 48 h, the 2.5, 5, and 10 µg methoxyfenozide doses produced between 40 and 60 picomols (Fig. 3.5).

2.5 Induction of autogeny using topically applied ecdysone or 20E. Ecdysone and 20E are known to stimulate yolk deposition in *Ae. aegypti*. Yolk deposition was seen in the 20E treated mosquitoes at 24 h and 48 h, but not in the ecdysone treated individuals. All doses of 20E had between 50 and 100 µm of yolk at 24 h and between 100 and 200 µm at 48 h (Fig 3.6). There was no trend in chymotrypsin-like serine protease activity levels (Fig 3.7). Ecdysteroid production for 20E was around 600 pg at 24h and between 200 and 400 pg at 48 h. For ecdysone, ecdysteroid levels were between 1000 and 1200 pg at 24 and 48 h (Fig 3.8).

2.6 Host seeking in females exhibiting autogeny after treatment with methoxyfenozide or an ecdysteroid. Mosquitoes that are autogenous for their first gonotropic cycle typically will not host seek or take a bloodmeal until they have oviposited. I wanted to determine whether or not females treated with methoxyfenozide and exhibiting yolk deposition would take a blood meal. Induction of autogeny by methoxyfenozide seemed to inhibit mosquitoes from seeking a host and taking a bloodmeal at any dose, except for the 20 µg dose, at which the females showed no interest in blood feeding (Table 3.14).

Effects of methoxyfenozide and tebufenozide on ecdysteroid production in vitro. This experiment was performed to determine if the lower number of eggs deposited by females treated with methoxyfenozide was due to effects on the ovaries. Ovaries from blood fed mosquitoes were incubated with methoxyfenozide and tebufenozide. Ovaries from non-blood fed mosquitoes were incubated with methoxyfenozide and tebufenozide to determine if the ecdysone

agonists would cause the ovaries to release ecdysone. Neither compound in vitro stimulated ecdysteroid production by ovaries of non-blood fed females (Fig 3.9, 3.10) or inhibited ecdysteroid production by ovaries of blood fed females taken at 24 h after a blood meal (Fig 3.11, 3.12).

Discussion

Similar to azadirachtin, females were unaffected by exposure to treated filter paper. Mortality was low regardless of whether the mosquitoes had been blood fed or not with one exception. Contact exposure also had no effect on the average number of eggs oviposited. Just as in the previous chapter, females that were exposed to impregnated filter paper suffered no ill effects. When filter paper is impregnated with a pure granular substance, it appears to have difficulty penetrating the insect cuticle.

Topical application of tebufenozide to blood fed mosquitoes was toxic at high doses but had no effect on the number of eggs oviposited, regardless of when the mosquito was treated relative to the blood meal. Topical application of methoxyfenozide was also toxic at high doses, but decreased the number of eggs oviposited, with females treated with 20 µg not ovipositing. Mosquitoes that did not oviposit were found to have fully yolked oocytes. A similar phenomenon was seen by Swever and Iatrou (1999) in *Bombyx mori*. When pupal *Bombyx mori* were treated with tebufenozide, the ovaries were arrested and would not develop the chorion of the egg. This was attributed to tebufenozide being able to stimulate vitellogenesis, but the inability to initiate choriogenesis. At this point in oogenesis in *Ae. aegypti* (48 h PBM), ecdysteroid titres would have returned to normal levels to allow late gene transcription, but methoxyfenozide would still be bound to the EcR/USP complex, due to not being easily metabolized by the insect, activating the receptor and not allowing late gene transcription.

OEH and ILP release from the head soon after blood ingestion stimulates the ovaries to produce ecdysteroid hormones. Ecdysone from the ovaries is converted into 20E in the fat body where, along with amino acids from the midgut, expression of the vitellogenin gene is stimulated (Raikhel et al., 2007). Tebufenozide and methoxyfenozide stimulate yolk deposition in decapitated blood fed females. This led me to determine that these ecdysteroid agonists also stimulate yolk deposition in non-blood fed females. In autogenous mosquitoes like *Georgcraigius (Ochlerotatus) atropalpus*, the fat body begins to produce vitellogenin in response to ecdysteroids produced by the ovaries without a blood meal (Telang et al., 2006).

Ge. atropalpus stores large amounts of amino acids in storage proteins as larvae for use as raw materials to make this vitellogenin (Wheeler et al. 1996). *Ge. atropalpus* breaks down these storage proteins in the fat body using the enzyme chymotrypsin, as opposed to trypsin, which is used to digest blood in the midgut of anautogenous mosquitoes (Masler et al., 1983). When *Ae. aegypti* females are treated with methoxyfenozide or tebufenozide, chymotrypsin-like serine protease activity is detected in the fat body, suggesting that the mosquito is attempting to mobilize stored resources in the fat body to produce vitellogenin, like *Ge. atropalpus*. Yolk deposition into the oocytes occurs and the content of the oocytes was confirmed to be vitellogenin using a western blot. Since the mosquitoes were developing oocytes, I checked to see if they would blood feed, with all of the treated females feeding freely except for the 20 µg treatment.

The comparison of methoxyfenozide to 20E and ecdysone suggest that methoxyfenozide stimulates vitellogenin production in the fat body in the same way as the ecdysteroids. Chymotrypsin-like serine protease activity of 5 µg of methoxyfenozide was equivalent to that of 20E and ecdysone. Yolk deposition was also equivalent between 20E and methoxyfenozide.

Ovary ecdysteroid production was dissimilar, with the highest level in methoxyfenozide being 60 pg at the 10 µg dose, and 20E and ecdysone being closer to 1000 pg. This suggests that while methoxyfenozide activates the EcR/USP complex, it does not create a feedback loop with the ovaries to keep producing ecdysone. This is further supported by Figs. 3.9 and 3.10 that show that methoxyfenozide does not stimulate or inhibit ecdysteroid production by the ovaries.

In vitro experiments demonstrated that methoxyfenozide and tebufenozide did not inhibit or stimulate ecdysteroid production in the ovaries of non-blood fed or blood fed mosquitoes. This suggests that methoxyfenozide and tebufenozide do not affect the ovaries directly, but acts on a different target within the signaling cascade, most likely the fat body, since the fat body is the usual target of endogenous ecdysteroids.

In the present study I showed that methoxyfenozide was toxic at high doses, but affected female mosquitoes physiologically at low doses, while tebufenozide only was toxic at high doses, but had no physiological effects. Methoxyfenozide could be used to kill mosquitoes, and if a lethal dose was not obtained, it would still impede egg production. Perhaps more interesting is the ability of methoxyfenozide to stimulate vitellogenesis. Further work should look at why methoxyfenozide does not stimulate ecdysteroid production by the ovaries, while 20E and ecdysone, the chemicals that it mimics, do stimulate ecdysteroid production.

Table 3.1: Cumulative mortality of non-blood fed *Ae. aegypti* with continuous exposure to tebufenozide.

	Ethanol	500 µg	1000 µg	5000 µg
N	10	10	10	10
Total dead at 24 h	0	0	0	0
Total dead at 48 h	0	0	0	0
Total dead at 72 h	0	0	0	0
Total dead at 96 h	0	2	0	0
Total dead at 120 h	0	2	1	8
Percent Mortality	0%	20%	10%	80%

Table 3.2: Cumulative mortality of non-blood fed *Ae. aegypti* with continuous exposure to methoxyfenozide.

	Ethanol	500 µg	1000 µg	5000 µg
N	10	10	10	10
Total dead at 24 h	0	0	0	0
Total dead at 48 h	0	0	0	0
Total dead at 72 h	0	0	0	0
Total dead at 96 h	0	0	0	0
Total dead at 120 h	0	0	0	0
Percent Mortality	0%	0%	0%	0%

Table 3.3: Cumulative mortality and average number of eggs oviposited by blood fed *Ae. aegypti* with continuous tebufenozide exposure for 48 h.

	Ethanol	500 µg	1000 µg	5000 µg
N	10	10	10	10
Total dead at 24 h	0	0	0	0
Total dead at 48 h	0	0	1	0
Total dead at 72 h	0	0	1	0
Total dead at 96 h	0	0	1	0
Percent Mortality pre oviposition	0%	0%	10%	0%
Percent Mortality post oviposition	30%	20%	40%	40%
Average egg #/ female (N)	98 (9)	104 (10)	108 (9)	94 (8)

Table 3.4: Cumulative mortality and average number of eggs oviposited by blood fed *Ae. aegypti* with continuous methoxyfenozide exposure for 48 h.

	0 µg	500 µg	1000 µg	5000 µg
N	10	10	10	10
Total dead at 24 h	0	0	0	0
Total dead at 48 h	0	0	0	0
Total dead at 72 h	0	0	0	0
Total dead at 96 h	0	0	0	0
Percent Mortality pre oviposition	0%	0%	0%	0%
Percent Mortality post oviposition	30%	60%	50%	50%
Average egg #/ female (N)	98 (9)	117 (9)	94 (10)	90 (10)

Table 3.5: Cumulative mortality of non-blood fed *Ae. aegypti* by topical application of tebufenozide.

	Ethanol	.25 µg	2.5 µg	5 µg	10 µg	20 µg
N	30	30	30	30	30	30
Total dead at 24h	0	0	1	1	0	1
Total dead at 48h	0	0	1	1	3	4
Total dead at 72h	1	3	3	2	3	6
Total dead at 96h	1	4	5	3	9	15
Total dead at 120h	6	9	9	5	13	21
Percent Mortality at 120	20%	30%	30%	17%	43%	70%

Table 3.6: Cumulative mortality of non-blood fed *Ae. aegypti* by topical application of methoxyfenozide.

	0 µg	.25 µg	2.5 µg	5 µg	10 µg	20 µg
N	30	30	30	30	30	30
Total dead at 24h	0	0	0	3	2	3
Total dead at 48h	0	2	0	7	6	6
Total dead at 72h	1	4	4	8	9	8
Total dead at 96h	1	8	8	18	23	23
Total dead at 120h	6	11	12	24	27	25
Percent Mortality at 120	20%	37%	40%	80%	90%	83%

Table 3.7: Effects of topical tebufenozide treatment 24 h before a blood meal on pre and post oviposition mortality and number of eggs oviposited in *Ae. aegypti*.

	0 µg	0.25 µg	2.5 µg	5 µg	10 µg	20 µg
N	80	80	80	80	80	80
Mortality pre oviposition	4%	11%	9%	13%	20%	36%
Mortality post oviposition	28%	41%	50%	51%	63%	76%
Average egg #/ female (N)	91 (50)	90 (57)	81 (45)	89 (59)	67 (40)	72 (17)

Table 3.8: Effects of topical methoxyfenozide treatment 24 h before a blood meal on pre and post oviposition mortality and number of eggs oviposited in *Ae. aegypti*.

	0 µg	0.25 µg	2.5 µg	5 µg	10 µg	20 µg
N	80	80	80	80	80	80
Mortality pre oviposition	4%	14%	11%	15%	30%	30%
Mortality post oviposition	28%	43%	40%	53%	64%	94%
Average egg #/ female (N)	91 (50)	89 (53)	53 (48)	21 (29)	2 (14)	1 (8)

Table 3.9: Percent mortality and number of eggs oviposited after being treated topically with tebufenozide 1 h after a blood meal.

	0 µg	0.25 µg	2.5 µg	5 µg	10 µg	20 µg
N	20	20	20	20	20	20
Mortality pre oviposition	10%	0%	10%	0%	20%	5%
Mortality post oviposition	30%	55%	55%	65%	75%	45%
Average egg #/ female (N)	96 (14)	72 (9)	86 (9)	94 (9)	102 (5)	73 (11)

Table 3.10: Percent mortality and number of eggs oviposited after being treated topically with methoxyfenozide 1 h after a blood meal.

	0 µg	0.25 µg	2.5 µg	5 µg	10 µg	20 µg
N	30	30	30	30	30	30
Mortality pre oviposition	20%	47%	43%	67%	57%	70%
Mortality post oviposition	47%	90%	90%	93%	97%	83%
Average egg #/ female (N)	107 (20)	57 (13)	28 (19)	11 (10)	0 (12)	21 (13)

Table 3.11: Cumulative mortality of larval *Ae. aegypti* with continuous exposure to methoxyfenozide.

	0 µg / 100 ml	10 µg / 100 ml	100 µg / 100 ml	1000 µg / 100 ml
N	50	50	50	50
Mortality at 24 h	0	0	15	27
Mortality at 48 h	8	12	43	46
Mortality at 72 h	9	14	50	50
Mortality at 96 h	13	14	50	50
% Mortality at 120h	26%	28%	100%	100%

Table 3.12: Effects of topical treatment with different amounts of tebufenozide on the average yolk deposition and mortality in non-blood fed, female, *Aedes aegypti* females.

Tebufenozide dose	0 µg	0.25 µg	2.5 µg	5 µg	10 µg	20 µg
N	35	35	35	35	35	35
% Mortality at 24 h	0%	0%	0%	2.8%	2.8%	0%
% Mortality at 48 h	0%	0%	0%	8.5%	8.5%	0%
Average Yolk deposition 24 h PBM (number treated)	0 (35)	3 (35)	34 (34)	45 (34)	44 (34)	25 (33)
Average Yolk deposition 48 h PBM (number treated)	0 (35)	9 (35)	36 (22)	69 (21)	116 (28)	64 (34

Table 3.13: The effects of different amounts of methoxyfenozide applied topically to non-blood fed, female *Aedes aegypti* on yolk deposition at 24 and 48 h post treatment.

Methoxyfenozide dose	0 µg	0.25 µg	2.5 µg	5 µg	10 µg	20 µg
N	55	55	55	55	55	55
% Mortality at 24 h	4%	4%	20%	6%	4%	5%
% Mortality at 48 h	4%	4%	29%	13%	21%	30%
Average Yolk Deposition 24 h PBM (number treated)	0 (53)	59 (52)	67 (45)	64 (51)	66 (52)	63 (42)
Average Yolk Deposition 48 h PBM (number treated)	0 (53)	65 (51)	127 (28)	143 (38)	122 (34)	142 (35)

Table 3.14: The effects of methoxyfenozide, ecdysone, and 20E on host seeking and receptivity to a blood meal

	Number of females that took a blood meal 24 h post treatment	Number of females that took a blood meal 48 h post treatment
N	**10**	**10**
Ethanol	8	9
0.05 µg 20E	8	0
0.5 µg 20E	6	2
1 µg 20E	6	1
2.5 µg 20E	7	2
5 µg 20E	8	3
0.25 µg ecdysone	0	1
0.5 µg ecdysone	4	3
1 µg ecdysone	2	2
2.5 µg Methoxyfenozide	3	1
5 µg Methoxyfenozide	1	0
10 µg Methoxyfenozide	3	0
20 µg Methoxyfenozide	0	1

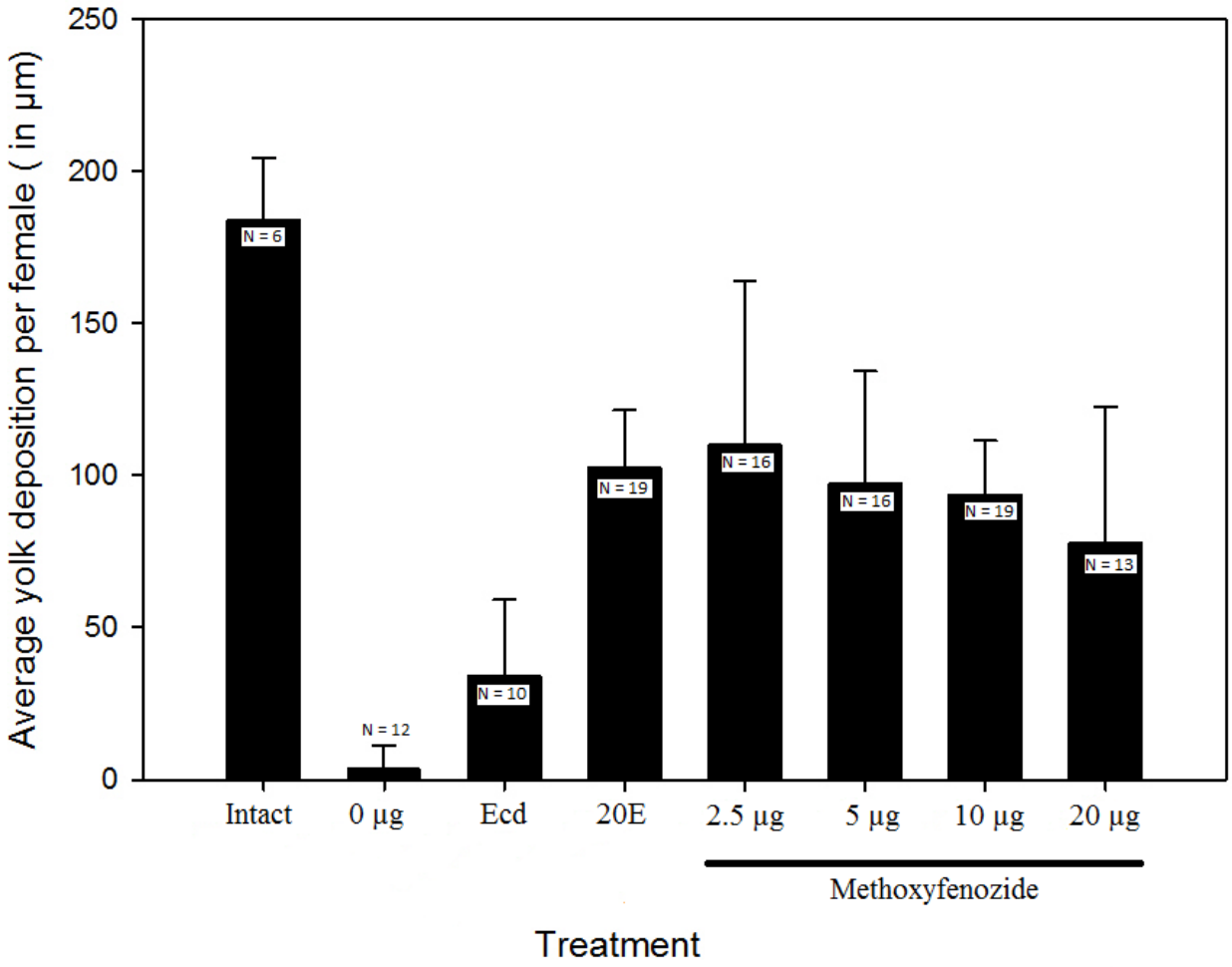

Fig 3.1: The effects of topical treatment with methoxyfenozide, ecdysone or 20E on yolk deposition in mosquitoes decapitated 1h post blood meal or left intact.

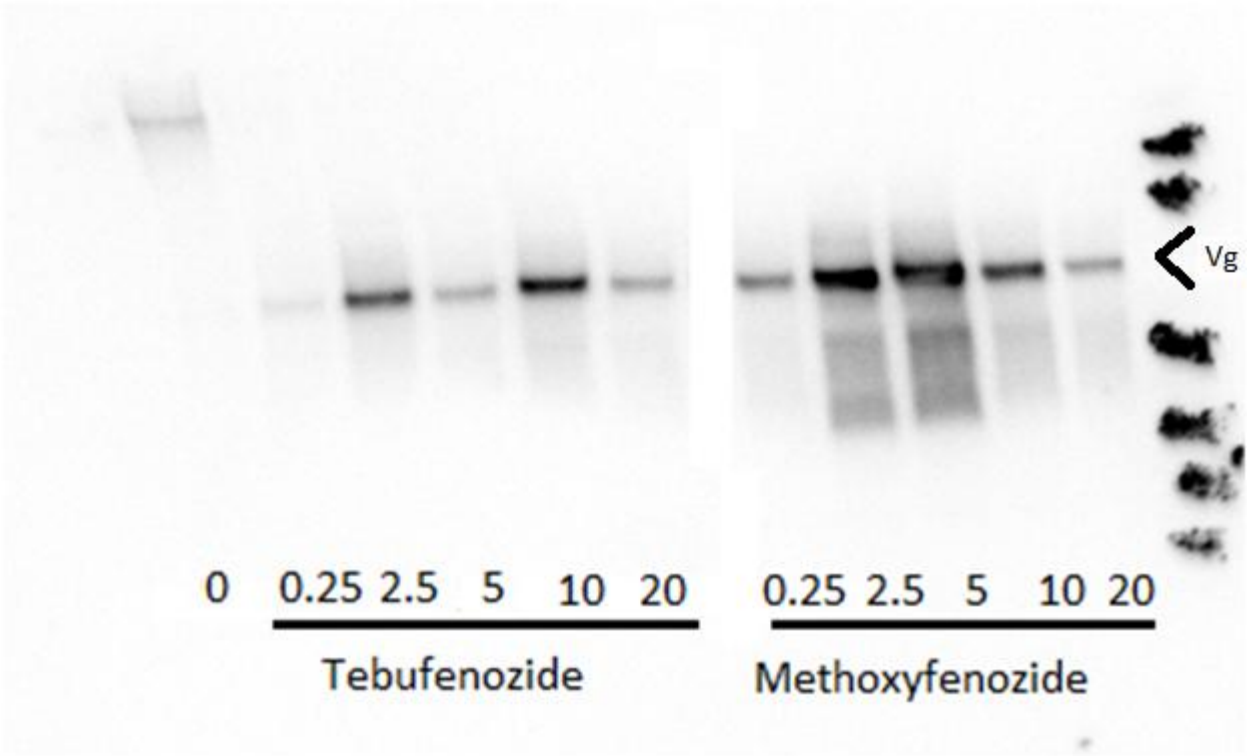

Fig. 3.2: Immunoblot of abdomens of non-blood fed female *Aedes aegypti* having been topically treated with either methoxyfenozide or tebufenozide. The bands seen are the 220 kDa vitellogenin band. Two abdomens per lane.

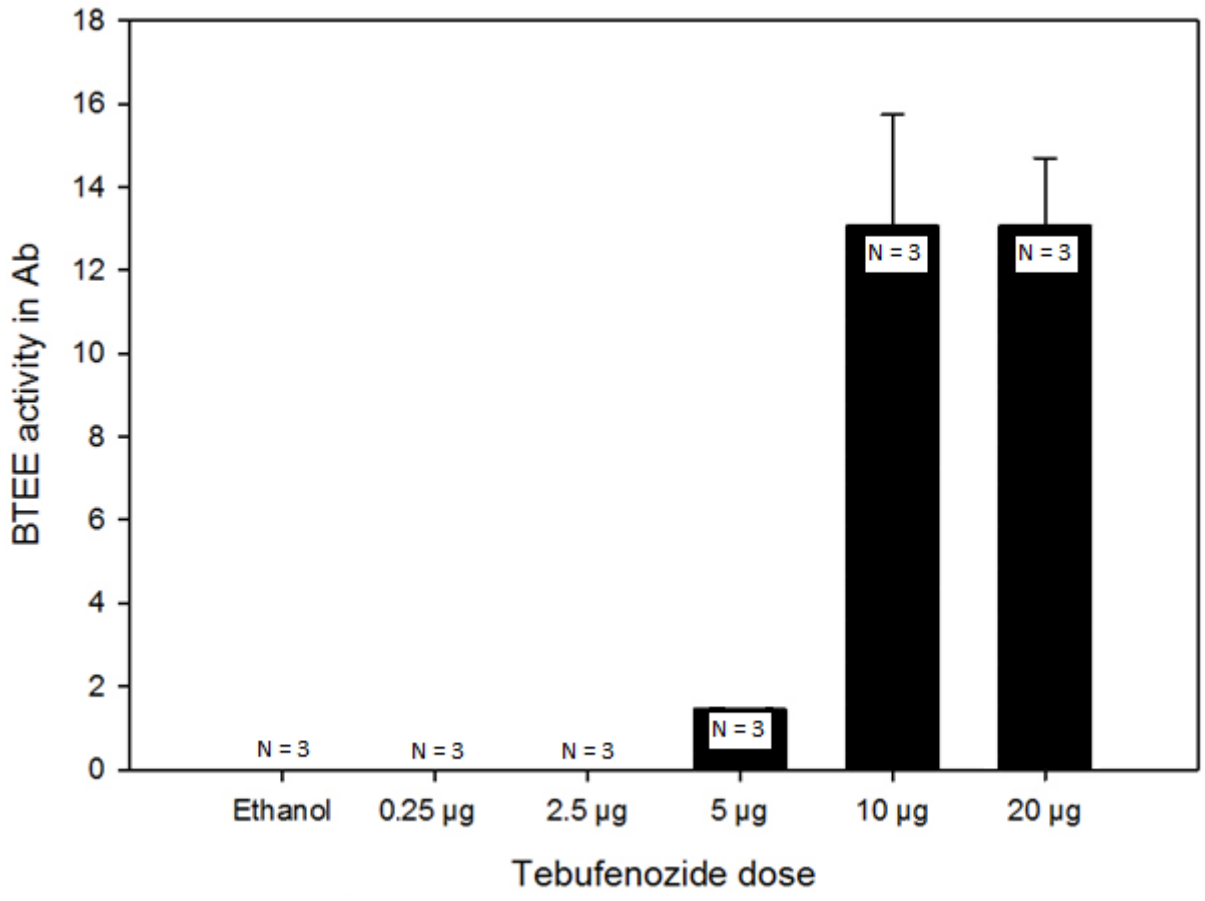

Fig. 3.3: The effect of topical treatment of different amounts of tebufenozide on chymotrypsin activity in the abdomen (minus the hindgut, midgut, and Malpighian tubules) of 3 day old, non-blood fed, female *Aedes aegypti*.

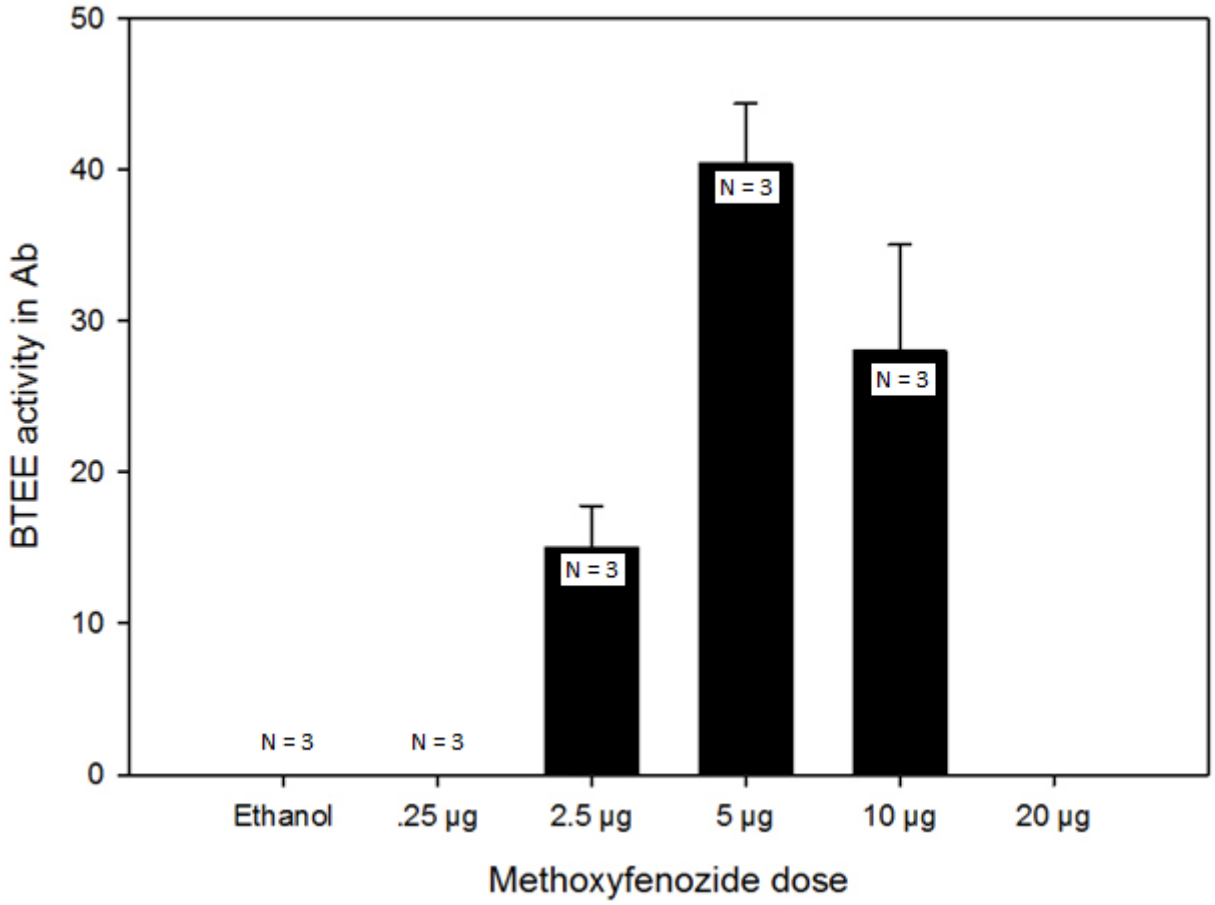

Fig.3.4: The effects of topical treatment of different amounts of methoxyfenozide on chymotrypsin-like activity in the abdomen (minus the hindgut, midgut, and Malpighian tubules) of 3 day old, non-blood fed, female *Aedes aegypti*.

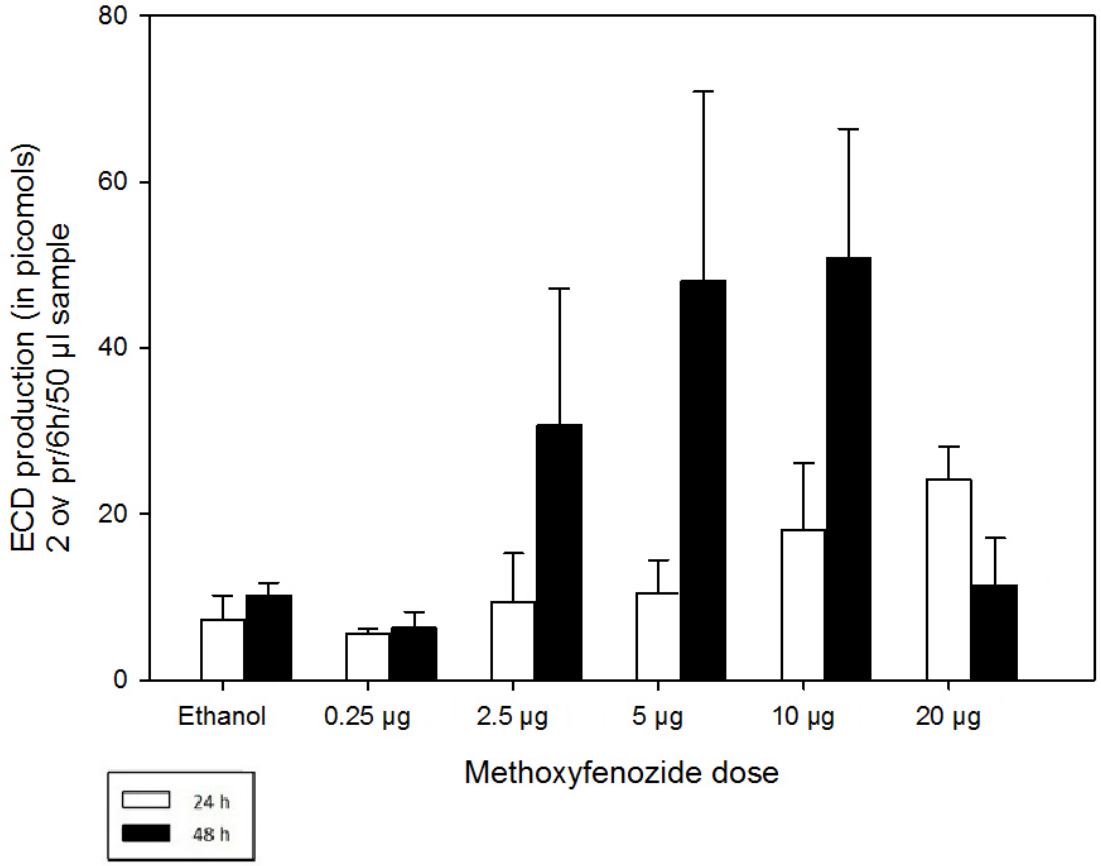

Fig. 3.5: The effects of methoxyfenozide treatment 24 h before dissection on ecdysteroid production by non-blood fed ovaries in *Ae. aegypti*. Ovaries were incubated for six hours and an ecdysteroid RIA was performed. Triplicate samples.

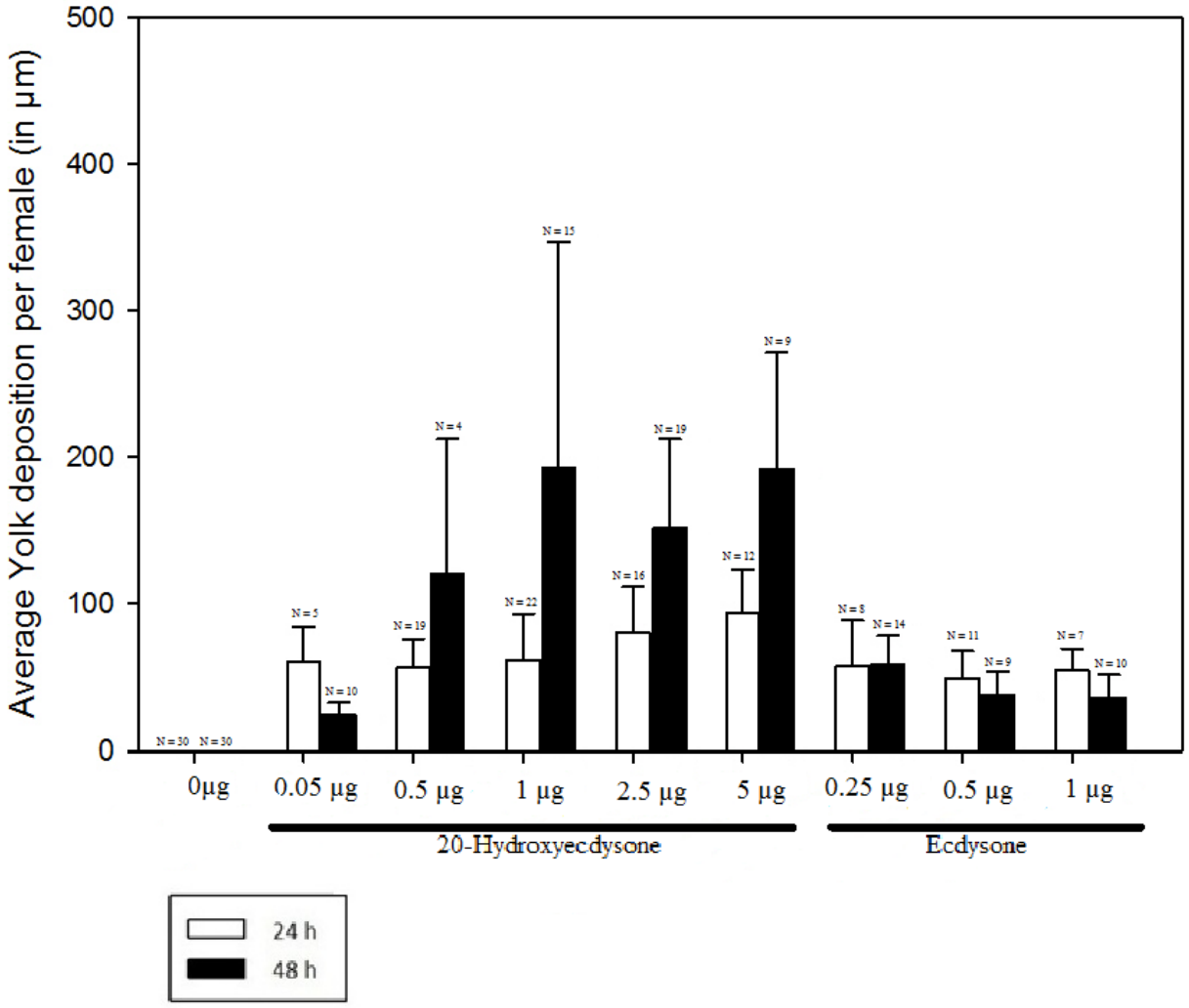

Fig. 3.6: The effects of different doses of 20E and ecdysone on yolk deposition in non-blood fed *Ae. aegypti*.

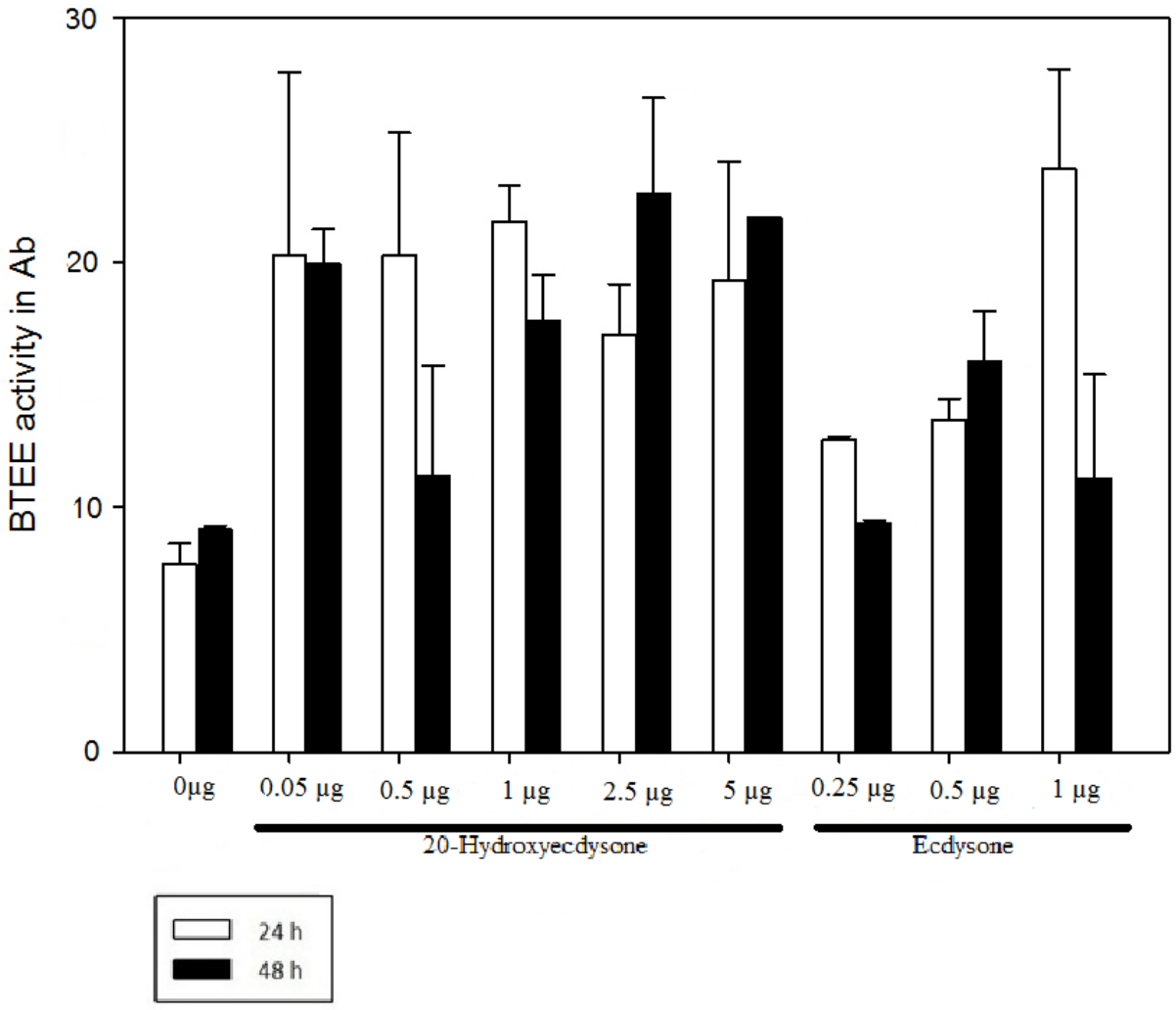

Fig 3.7: The effects of different doses of 20E and ecdysone on chymotrypsin-like serine protease activity in non-blood fed female *Ae. aegypti.*

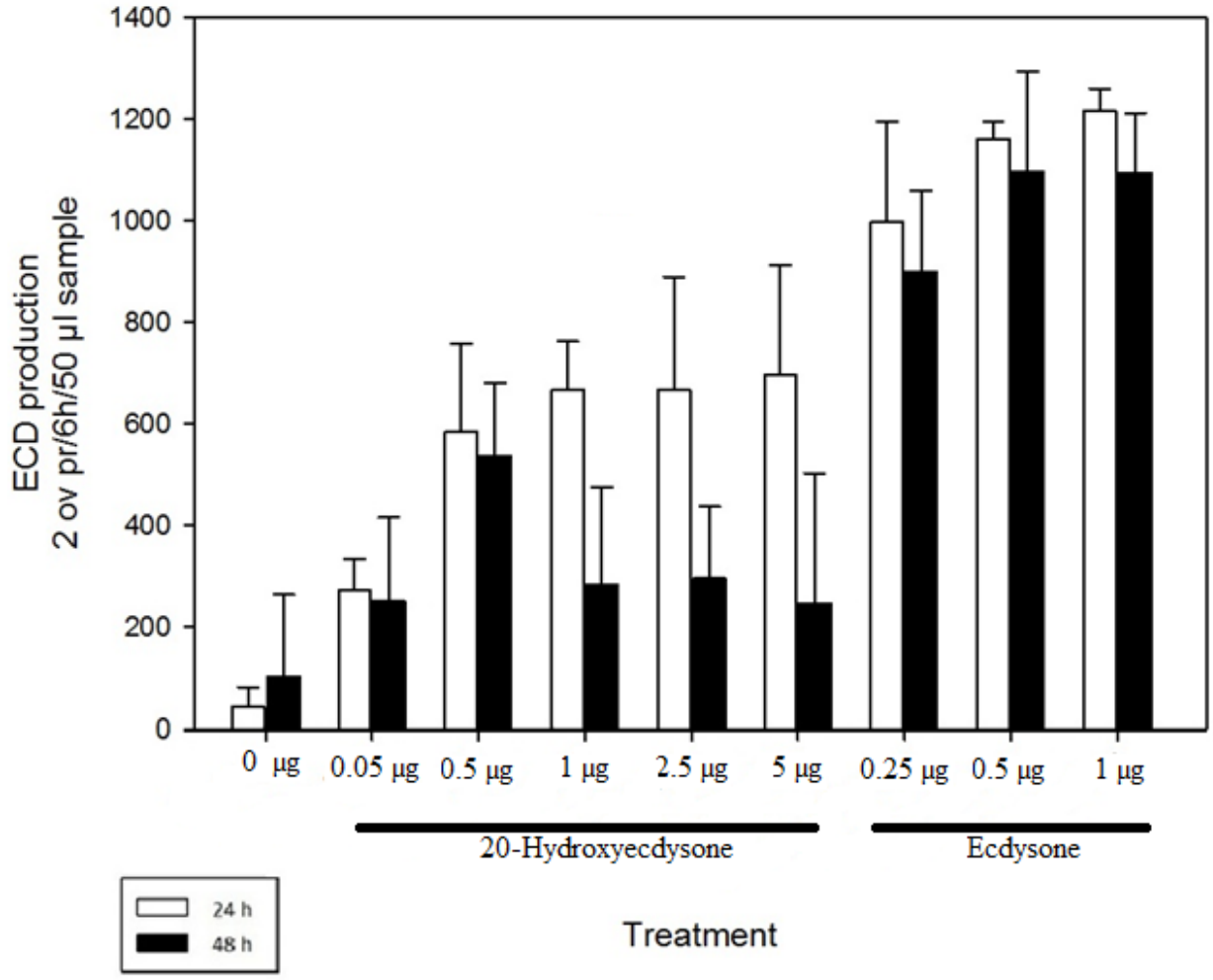

Fig. 3.8: The effects of different doses of 20E and ecdysone on ecdysteroid production in non-blood fed *Ae. aegypti*.

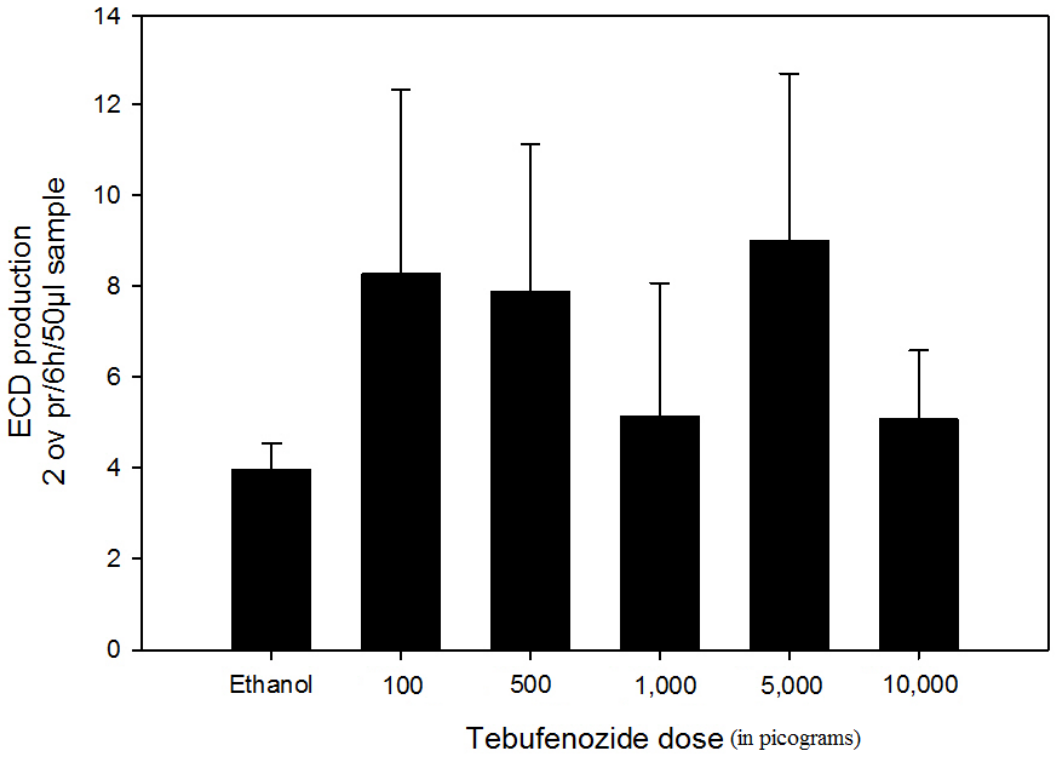

Fig 3.9: The effects of different amounts of tebufenozide, incubated with non-blood fed ovaries for six hours, on ecdysteroid secretion. All samples are triplicate samples.

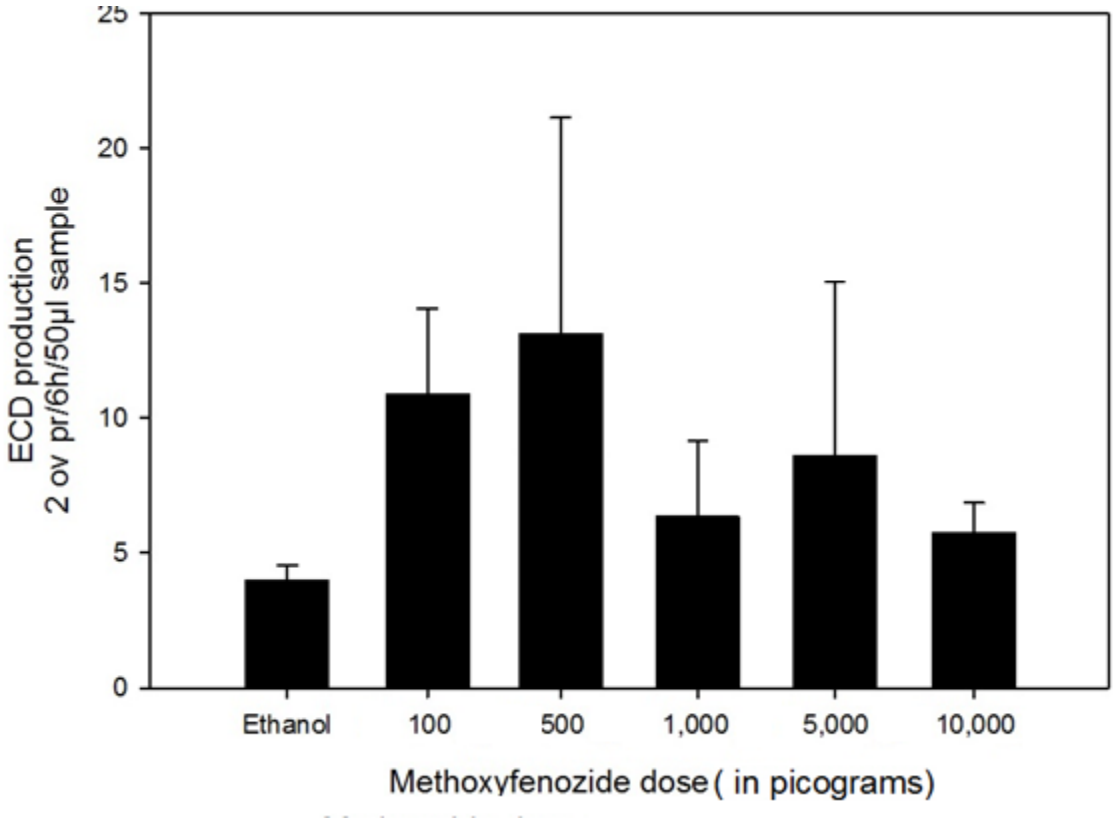

Fig 3.10: The effects of different amounts of methoxyfenozide, incubated with non-blood fed ovaries for six hours, on ecdysteroid secretion. All samples are triplicate samples.

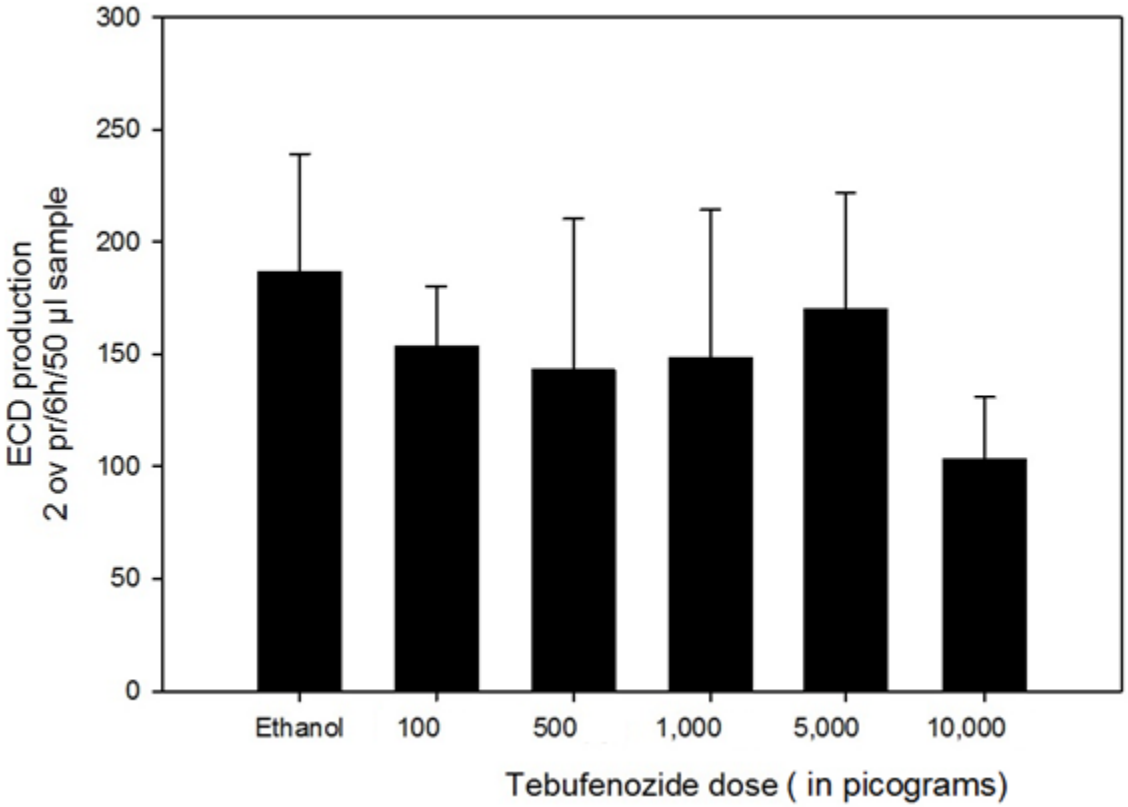

Fig. 3.11: The effects of different amounts of tebufenozide, incubated with 24 h blood fed ovaries for six hours, on ecdysteroid secretion. All samples are triplicate samples.

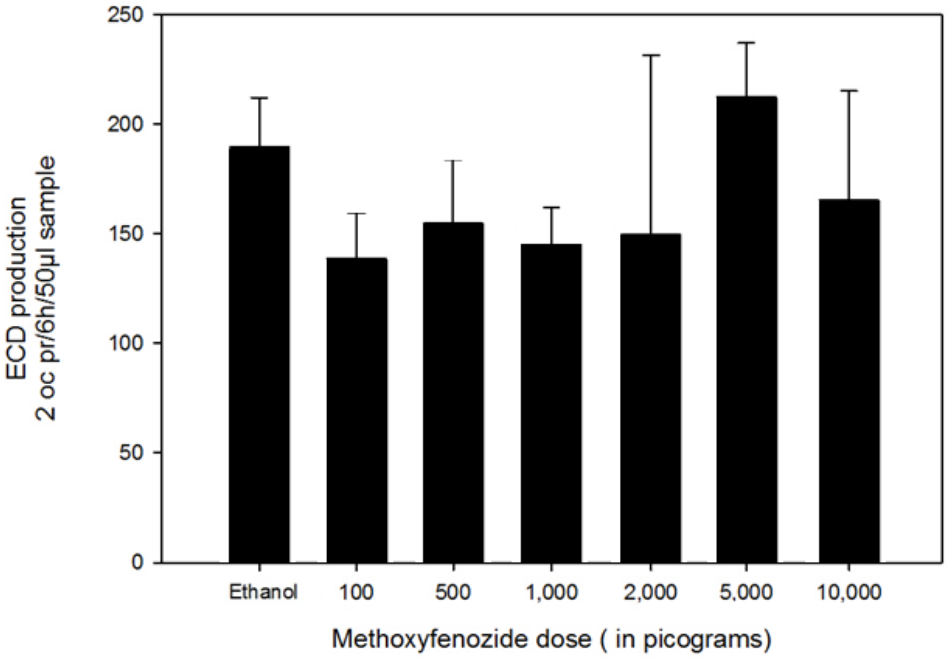

Fig. 3.12: The effects of different amounts of methoxyfenozide, incubated with 24 h blood fed ovaries for six hours, on ecdysteroid secretion. All samples are triplicate samples.

CHAPTER 4

CONCLUSIONS

Mosquitoes typically require the amino acids derived from a blood meal from a vertebrate host to be able to complete egg maturation and reproduce. Repeated hematophagy makes mosquitoes an ideal vector for many blood borne pathogens that are responsible for a large number of deaths every year. In an effort to reduce the instance of many of these diseases, mosquito control programs have focused on removing the mosquito vectors of these diseases using insecticides.

Many insecticides that were successful at controlling pest populations in the past have negative, unintentional consequences on the environment. These range from toxicity to non-target animals, persistence in the environment, and even the possibility of breaking down into more toxic chemicals. Plant botanicals and IGRs are two classes of insecticides that have been developed as safer alternatives to organic synthetic insecticides. They address ecological concerns by being specific to insects, having low retention times in the environment, and breaking down into relatively harmless compounds.

In this study, I examined what effects the plant botanical azadirachtin and the IGRs methoxyfenozide and tebufenozide had on the mortality and reproduction of female *Ae. aegypti*. Additionally, I tested whether the agonistic qualities of methoxyfenozide would stimulate egg maturation in decapitated blood fed mosquitoes as well as intact non-blood fed mosquitoes.

I determined that topical application of the three compounds was the most effective delivery method for killing adult stage *Ae. aegypti*, while feeding of intermediate doses of

azadirachtin reduced the number of eggs laid per female. Overall, my results suggested that azadirachtin is not a useful insecticide for control of adult *Ae. aegypti*.

I found that tebufenozide was toxic at the highest dose tested. However, treatment with tebufenozide caused no sublethal effects on the mosquitoes. Methoxyfenozide was toxic and high doses also caused females to lay fewer eggs or not oviposit at all. Methoxyfenozide was also able to stimulate yolk deposition into the ovaries of non-blood fed mosquitoes. The nutrients for this yolk came from the breakdown of the fat body. These females never finished egg maturation or oviposited eggs.

REFERENCES

1. **Adelman, Z., N. Jasinskiene, and A. James. 2002.** Development and applications of transgenesis in the yellow fever mosquito, *Aedes aegypti*. Mol Biochem Parasit 121: 1-10
2. **Attardo, G., I. Hansen, and A. Raikhel. 2005.** Nutritional regulation of vitellogenesis in mosquitoes: implications for autogeny. Insect Biochem Molec. 35: 661-675
3. **Azambuja, P., E. Garcia, N. Ratcliffe, and D. Warthen. 1991.** Immune-depression in *Rhodnius prolixus* induced by the growth inhibitor, Azadirachtin. J Insect Physiol. 10: 771-777.
4. **Barnby, M., and J. Klocke. 1990.** Effects of azadirachtin on levels of ecdysteroids and prothoracicotropic hormone-like activity in *Heliothis virescens* larvae. J Insect Physiol. 36: 125-131
5. **Beckage N., K. Marion, W. Walton, M. Wirth, and F. Tan. 2004.** Comparative larvicidal toxicities of three ecdysone agonists on the mosquitoes *Aedes aegypti*, *Culex quinquefasciatus*, and *Anopheles gambiae*. Arch Insect Biochem. 57: 111-122.
6. **Bidmon H, Kauser G, Mobus P, Koolman J**. **1987.** Effect of azadirachtin on blowfly larvae and pupae. 3rd Int. Neem Conference. 232-272.
7. **Boschitz, C., and J. Grunewald. 1994.** The effect of NeemAzal on *Aedes aegypti.* Appl Parasitol. 35: 251-256.

8. **Brown, M., R. Graf, K. Swiderck, D. Fendley, T. Stracker, D. Champagne, and A. Lea. 1998.** Identification of a steroidogenic neurohormone in female mosquitoes. J Biol Chem. 273: 3967-3971.
9. **Brown, M., K. Clark, M. Gulia, Z. Zhao, S. Garczynski, J. Crim, R. Suderman, and M. Strand. 2008.** An insulin-like peptide regulates egg maturation and metabolism in the mosquito *Aedes aegypti*. P Natl Acad Sci USA. 105: 5716-5721.
10. **Butterworth, J., and E. Morgan. 1968**. Isolation of a substance that suppresses feeding in locusts. J Chem Soc Chem Comm. 1: 23-24.
11. **Butterworth, J., and E. Morgan. 1971**. Investigation of the locust feeding inhibition of the seeds of the neem tree, Azadirachta *indica*. J Insect Physiol. 17: 969-977.
12. **Clements, A. 1992.** The Biology of Mosquitoes: Development, Nutrition and Reproduction. Chapman & Hall, London
13. **Dhadialla, T., A. Retnakaran, and G. Smagghe. 2005.** Insect growth- and development-disrupting insecticides. In Sarjeet S. Gill; Gilbert, Lawrence I.; Kostas Iatrou. Comprehensive molecular insect science. Amsterdam: Elsevier. pp. 55–115.
14. **Fallon, A., H. Hagedorn, G. Wyatt, and H. Laufer. 1974.** Activation of vitellogenin synthesis in the mosquito *Aedes aegypti* by ecdysone. J Insect Physiol 20: 1815-1823
15. **Graf, J. 1993.** The role of insect growth regulators in arthropod vontrol. Parasitol Today 912: 471-474.
16. **Gordon, R., and I. Burford. 1983.** Effects of methoprene, a juvenile hormone analogue, on the larval and pupal stages of the yellow fever mosquito, *Aedes aegypti*. J Insect Physiol. 30:279-286.

17. **Gulia-Nuss, M., A. Robertson, M. Brown, and M. Strand. 2011.** Insulin-like peptides and the target of rapamycin pathway coordinately regulate blood digestion and egg maturation in the mosquito *Aedes aegypti*. Plos One
18. **Hagedorn, H., J. Oconnor, M. Fuch, B. Sage, D. Schlaeger, and M. Bohm. 1975.** The ovary as a source of alpha ecdysone in an adult mosquito. P Natl Acad Sci USA. 72: 3255-3259.
19. **Hansen, I., G. Attardo, J. Park, Q. Peng, and A. Raikhel. 2004.** Target of Rapamycin-mediated amino acid signaling in mosquito anautogeny. P Natl Acad Sci USA 101: 10626-10631.
20. **Haskell, P., and A. Mordue-Luntz**. **1969.** The role of mouthpart receptors in the feeding behaviors of *Schistocera* gregaria. Entomol Exp Appl 12: 423-440.
21. **Howard A, E. Adongo, A. Hassanali, F. Omlin, A. Wanjoya, G. Zhou, and J. Vulule. 2009.** Laboratory evaluation of the aqueous extracts of *Azadirachta indica* (neem) wood chippings on *Anopheles gambiae* mosquitoes. J Med Entomol 46: 107-114.
22. **Hsu, A. 1991.** 1,2-Diacyl-1-alkyl-hydrazines; a novel class of insect growth regulators. In: Baker, D.R., Fenyes, J.G., Moberg,W.K. Eds., Synthesis and Chemistry of Agrochemicals, II. ACS Symposium Series, vol. 443. American Chemical Society, pp. 478–490.
23. **Jansen, C., and N. Beebe. 2010.** The dengue vector *Aedes aegypti*: what comes next. Microbes Infect. 12:272-279.

24. **Koolman, J., H. Bidmon, M. Lehmann, and G. Kauser** . **1988.** On the mode of action of azadirachtin in blowfly larvae and pupae. Endocrinological frontier in physiological insect ecology (Eds Sehnal F, Zabza A, and Denlinger D) vol. 1, pp55-67 Wroclaw technical univ. press, Wroclaw
25. **Koul, O., and M. Isman**. **1991.** Effects of azadirachtin on the dietary utilization and development of the variegated cutworm *Peridroma saucia*. J Insect Physiol. 37: 591-598.
26. **Klowden M. 1987.** Distention-mediated egg maturation in the mosquito, *Aedes aegypti*. J Insect Physiol. 33: 83-87.
27. **Lea, A. 1981.** Artificial stimulation of vitellogenesis in *Aedes aegypti* by 20-hydroxyecdysone. J Insect Physiol. 28: 173-176
28. **Linton, Y., A. Nisbet, and A. Mordue-Luntz. 1997.** The effects of azadirachtin on the testes of the desert locust, *Schistocerca gregaria* (Forskal). J Insect Physiol. 11: 1077-1084.
29. **Ludlum, C., and K. Sieber. 1988.** Effects of azadirachtin on oogenesis in *Aedes aegypti*. Physiol Entomol. 13: 177-184.
30. **Masler, E., L. Whisenton, D. Schlaeger, S. Kang, and M. Fuchs. 1983.** Chymotrypsin and trypsin levels in adult *Aedes atropalpus* and *Toxorhynchites brevipalpis*. Comp Biochem Physiol. 3: 435-440
31. **Meurant, K., C. Sernia, and H. Rembold. 1994.** The effects of azadirachtin-A on the morphology of the ring complex of *Lucilia cuprina* (Wied.) larvae (Diptera, Insecta). Cell Tissue Res. 275: 247-254.

32. **Mordue-Luntz, A., and A. Blackwell. 1993.** Azadirachtin: an update. J Insect Physiol. 11: 903-924

33. **Mordue-Luntz, A., E. Morgan, and A. Nisbet**, **2005.** Azadirachtin, a natural product in insect control, in comprehensive molecular insect science, edited by L. Gilbert, K. Iatrou and S. S. Gill, Elsevier BV, Amsterdam, 6: 117-135.

34. **Mulla, S., and T. Su. 1999.** Activity and biological effects of neem products against arthropods of medical and veterinary importance. J Am Mosquito Contr. 15: 133-152.

35. **Nathan, S., K. Kalaivani, and K. Murugan. 2005.** Effects of neem limonoids on the malaria vector *Anopheles stephensi* Liston. Acta trop. 96: 47-55.

36. **Radke, E., C. Gregory, K. Kintzinger, E. Sauber-Schatz, E. Hunsperger, G. Gallagher, J. Barber, and B. Biggerstaff. 2012.** Dengue outbreak in Key West, Florida, USA, 2009. Dispatch. 18

37. **Raikhel, A., V. Kokoza, J. Zhu, D. Martin, S. Wang, C. Li, G. Sun, A. Ahmed, N. Dittmer, and G. Attardo. 2002.** Molecular biology of mosquito vitellogenesis: from basic studies to genetic engineering of antipathogen immunity. Insect Biochem Molec. 32: 1275-1286

38. **Raikhel, A., and A. Lea. 1990.** Juvenile hormone controls previtellogenic proliferation of ribosomal RNA in the mosquito fat body. Gen Comp Endocr. 77: 423-434

39. **Raikhel, A., and A. Lea. 1983.** Previtellogenic development and vitellogenin synthesis in the fat body of a mosquito: an ultrastructural and immunocytochemical study. Tissue Cell. 15: 281-299

40. **Raizada, R., M. Srivastava, R. Kaushal, and R. Singh**. **2001.** Azadirachtin, a neem biopesticide: subchronic toxicity assessment in rats. Food Chem Toxicol. 39: 477-483.

41. **Redfern, R., T. Kelly, A. Borkovec, and D. Hayes. 1982.** Ecdysteroid titers and molting aberrations in last-stage *Oncopeltus* nymphs treated with insect growth regulators. Pestic Biochem Phys. 18: 351-356

42. **Retnakaran, A., J. Granett, and T. Ennis**. **1985.** Insect growth regulators. comprehensive insect physiology, biochemistry, and pharmacology. G. A. Kerkut, Gilbert, L.I. Oxford, Pergamon. 12.

43. **Riehle, M., Y. Fan, C. Cao, and M. Brown**. **2006.** Molecular characterization of insulin-like peptides in the yellow fever mosquito, *Aedes aegypti:* Expression, cellular localization, and phylogeny. Peptides. Doi:10.1016/j.peptides.2006.07.016

44. **Roy, S., I. Hansen, and A. Raikhel. 2007.** Effect of insulin and 20-hydroxyecdysone in the fat body of the yellow fever mosquito, *Aedes aegypti*. Insect Biochem Molec. 37: 1317-1326.

45. **Ruscoe C. 1972.** Growth disrupting effects of an insect antifeedant. Nature New Biol. 236: 159-160.

46. **Shaalan, E., D. Canyon, M. Younes, H. Abdel-Wahab, and A. Mansour. 2005.** A review of botanical phytochemicals with mosquitocidal potential. Environ Int. 31: 1149-1166.

47. **Siddall, J. 1976.** Insect Growth Regulators and Insect Control: A Critical Appraisal. Environ Health Persp. 14: 119-126.

48. **Sieglaff, D., K. Adams, and M. Brown. 2005.** Expression of genes encoding proteins involved in ecdysteroidogenesis in the female mosquito, *Aedes aegypti*. Insect Biochem Molec. 35: 471-490.

49. **Simmonds, M., and W. Blaney**. **1984.** Some effects of azadirachtin on lepidopterous larvae. Proceedings of the 2nd international neem conferences: 163-180.

50. **Smagghe, G., T. Dhadialla, and M. Lezzi**. **2002.** Comparative toxicity and ecdysone receptor affinity of non-steroidal ecdysone agonists and 20-hydroxyecdysone in *Chironomus tentans*. Insect Biochem Molec. 32: 187-192.

51. **Smith, S., and M. Mitchell. 1988.** Effects of azadirachtin on insect cytochrome P-450 dependent ecdysone 20-monooxygenase activity. Biochem Bioph Res Co 154: 559-563.

52. **Spielman, A., R. Gwadz, and W. Anderson. 1971.** Ecdysone initiated ovarian development in mosquitoes. J Insect Physiol. 17: 1807-1814.

53. **Staal, G. 1975.** Insect growth regulators with juvenile hormone activity. Annu Rev Entomol 20: 417-460.

54. **Staal, G. 1982.** Insect control with growth regulators interfering with the endocrine system. Entomol. Exp. Appl. 31: 15-23.

55. **Subrahmanyam, B., T. Muller, and H. Rembold. 1989.** Inhibition of turnover of neurosecretion by azadirachtin in *Locusta migratoria.* J Insect Physiol. 35: 493-497.

56. **Sun, X., Q. Song, and B. Barrett. 2003.** Effects of ecdysone agonists on the expression of EcR, USP and other specific proteins in the ovaries of the codling moth (*Cydia pomonella* L.) Insect Biochem Molec. 33: 829-840.

57. **Sun, X., Q. Song, B. Barrett. 2003.** Effects of ecdysone agonists on vitellogenesis and the expression of EcR and USP in Codling Moth (*Cydia pomonella*) Arc Insect Biochem. 52: 115-129.

58. **Sundaram, M., S. Palli, P. Krell, S. Sohi, T. Dhadialla, and A. Retnakaran. 1998.** Basis for selective action of a synthetic molting hormone agonist RH-5992 on lepidopteran insects. Insect Biochem Molec. 28: 693-704.

59. **Swevers, L., and K. Iatrou**. **1999.** The ecdysone agonist tebufenozide (RH-5992) blocks the progression into the ecdysteroid-induced regulatory cascade and arrests silkmoth oogenesis at mid-vitellogenesis. Insect Biochem Molec. 19: 955-963

60. **Telang, A., Y. Li, F. Noriega, and M. Brown**. **2006.** Effects of larval nutrition on the endocrinology of mosquito egg development. J. Exp Biol. 209: 645-655.

61. **Tolle, M**. **2009.** Mosquito-borne diseases. Curr Probl Pediatr Adolesc Health Care. 39: 97-140.

62. **Wandscheer, C., J. Duque , M. da Silva, Y. Fukuyama, J. Wohlke, J. Adelmann, and J. Fontana. 2004.** Larvicidal action of ethanolic extracts from fruit endocarps of *Melia azedarach* and *Azadirachta indica* against the dengue mosquito *Aedes aegypti*. Toxicon 829-835.

63. **Wheeler, D., and N. Buck. 1996.** A role for storage proteins in autogenous reproduction in *Aedes atropalpus*. J Insect Physiol. 42: 961-966.

64. **Wen, Z., M. Gulia, K. Clark, A. Dhara, J. Crim, M. Strand, and M. Brown. 2010.** Two insulin-like peptide family members from the mosquito *Aedes aegypti* exhibit different biological and receptor binding activities. Mol Cell Endocrinol. 328: 47-55.