ESTERASE ISOLATION, EXPRESSION, AND POPULATION ANALYSES OF *CULEX NIGRIPALPUS* Theobald (DIPTERA: CULICIDAE) OF MANATEE COUNTY, FL

By

SHAINNEL EANS

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2009

To all who supported and advised me throughout this project, thus contributing to my success

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I would like to thank the Manatee County Mosquito Control District and the Florida Department of Agriculture and Consumer Services for their support and funding of this project. I thank my committee members for their support and advice throughout my time working on this project. I also thank my mother for being my greatest inspiration throughout my studies. Without our combined efforts, this project would not have been possible.

LIST OF FIGURES

Abstract of Thesis Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Master of Science

ESTERASE ISOLATION, EXPRESSION, AND POPULATION ANALYSES OF *CULEX NIGRIPALPUS* Theobald (DIPTERA: CULICIDAE) OF MANATEE COUNTY, FL

By

Shainnel Eans

August 2009

Chair: Chelsea Smartt
Major: Entomology and Nematology

Mosquitoes play an important role as vectors for a wide variety of pathogens that cause diseases, including malaria, encephalitis, West Nile, dengue, and dengue hemorrhagic fever. In an attempt to control mosquito-borne disease outbreaks around the world, the use of insecticides has risen; however, this has also resulted in selection for mosquitoes that possess high tolerance/resistance to many insecticides. It is important to understand the mechanisms involved with the development of mosquito resistance to insecticides to be able to predict where targeted control measures may be needed and how mosquitoes will react to new insecticides. Since esterase is an enzymatic protein known to play a role in insecticide resistance formation, we amplified an esterase gene segment (Temsha est-1, TE-1) from *Culex nigripalpus* using esterase primers. Through expression studies, we found that TE-1 consistently showed high expression within thoraces and abdomens in unfed mosquitoes and high expression in heads, thoraces, abdomens, and midguts after blood feeding. This suggests TE-1 has a role in feeding/digestion. We also found that the level of expression of TE-1 differed depending on where the mosquitoes were collected. If this difference in expression can be correlated to differences in susceptibility towards insecticides, we may be able to use TE-1 as an indicator for the formation of tolerance/resistance. This would greatly enhance mosquito control efforts.

CHAPTER 1
INTRODUCTION

Mosquitoes serve as important vectors for a large array of pathogens that cause diseases including the following: malaria, encephalitis, West Nile, dengue, and filariasis (Greenwood 2002, Kyle and Harris 2008). Temperate and tropical areas worldwide are constantly faced with the threat of outbreaks of these diseases since mosquito populations thrive year-round in these regions (Clements 2000). This has lead to widespread usage of insecticides in order to help maintain control over mosquito populations (Djogbénou et al. 2009). The downside, however, is that this has also lead to populations of mosquitoes that have acquired resistance towards many insecticides (Levy 2007). For this reason we must learn to have a better understanding of the mechanisms involved with the development of mosquito resistance towards insecticides so that we can foresee what control measures could prove to be more effective and so that we can better predict how mosquitoes would react to newly formulated insecticides. It is beneficial to counteract or at least minimize the defenses mosquitoes have towards insecticides by directly manipulating the genes that control the resistance phenotype (Kidwell and Wattam 1998).

In the United States, for example, the distribution of mosquito-vectored diseases (such as West Nile) has caused distress due to the impact they have on public health in the event of an outbreak (Hayes 2005). In addition, control of vectors of West Nile virus (WNV), such as *Culex* mosquitoes in the southeastern United States, has become economically significant due to the expenses involved in maintaining effective control (Sardelis et al. 2001, Huhn 2003, Rutledge et al. 2003, Djogbénou et al. 2009). States such as Florida, in particular, are prime targets of concern regarding potential outbreaks of mosquito-vectored diseases, such as West Nile, due to their geographic location and the warm humid climate that favors the growth of mosquito populations throughout the year (Nayar 1982).

An intuitive method to control the continued spread of WNV, along with any other pathogens that are transmitted by mosquitoes, is to maintain control over the mosquito population number. This is a good control strategy, because mosquitoes are essential for the pathogen's continued transmission and survival (Hemingway and Ranson 2000). Mosquito-control programs are confronted with the task of developing effective control measures that reduce the ability of mosquito populations to spread diseases to human populations (Perera 2008).

Since the mid 1900's the United States has developed several programs aimed towards mosquito control to reduce mosquito populations and to reduce their ability to transmit disease (Levy 2007). The usage of insecticides increased as an effective control strategy for this purpose since the mid-1950s (Djogbénou et al. 2009). Initially, these control strategies involving insecticides preferentially used the toxin known as DDT (Dichloro-Diphenyl-Trichloroethane) (WHO 1979, Coleman and Hemingway 2007). Such extensive usage of DDT was successful at first, but it soon resulted in the development of resistance within mosquito populations (WHO 1979, Levy 2007). With the effectiveness of DDT on the decline, along with knowledge of its negative environmental impacts on local plants, animals, and people, mosquito control programs began to use other types of insecticides (Guessan et. al. 2007). These other insecticides included carbamates and organophosphates (such as malathion, chloropyrifos, naled, propoxor, and fenthion) and pyrethroids (such as resmethrin and permethrin) (WHO 1979).

Organophosphates and carbamates both share a similar mode of action, which is to prevent nerve impulses from transmitting properly between the synapses of mosquito nerve cells (Dent 1995, Brown 2006). Organophosphates are chemically less stable, meaning they have a short timeframe after application in which they remain effective before breaking down in the

environment. The timing of applications of organophosphates is thus important to consider (Dent and Elliot 1995). Organophosphate insecticides have a potent toxicity towards both their intended targets and mammals. The effect of organophosphates is often irreversible (Brown 2006). Although carbamates share a similar mode of action to organophosphates, the toxic effects of carbamates are more easily reversed than that of organophosphates if the appropriate dosage is not taken up by their intended targets (Dent 1995).

DDT and pyrethroids also share a similar mode of action, which is to modify voltage-gated ion channels within the nerve cells of targeted mosquitoes (Brown 2006). DDT is a chlorinated hydrocarbon, also called an organochlorine insecticide. DDT is a long-lasting and stable insecticide that has been in use since the mid-1900s in the United States and many other countries throughout the world; however, due to the build-up of resistance there has been a shift towards using pyrethroids, carbamates and organophosphates instead (http://ipmworld.umn.edu/chapters/curtiscf.htm). Pyrethroids are more potent in smaller dosages than DDT and they are longer lasting and more easily applied on a wider range of surfaces (such as mud walls) than organophosphates or carbamates (http://ipmworld.umn.edu/chapters/curtiscf.htm).

DDT and pyrethroids have a similar mode of action, thus in regions where DDT resistance has been detected (such as in Sri Lanka, India, Pakistan, Turkey, and Central America) there is an emphasis on using organophosphates and carbamates (http://ipmworld.umn.edu/chapters/curtiscf.htm). However, due to the relatively shorter longevity and higher production costs of organophosphates and carbamates, DDT and pyrethroid insecticides are still used in many countries (http://ipmworld.umn.edu/chapters/curtiscf.htm). In Florida, DDT is no longer used (Florida Coordinating Council on Mosquito Control 1998).

Although in the mid-1900s, DDT was commonly used in Florida mosquito control programs, currently pyrethroids, carbamates, and organophosphates are used (http://edis.ifas.ufl.edu/PI172).

Mosquitoes display a resistance or tolerance towards insecticides as they become increasingly less vulnerable to the toxicity. Although resistance and tolerance are often used interchangeably there are slight differences between the two terms that must be kept in mind. We use the term "tolerance" to refer to the mosquito's ability to endure or become less responsive to a substance with repeated exposure; while we use the term "resistance" to refer to the ability of the mosquito to form immunity towards an insecticide after exposure. Why is it that a given insecticide can show high effectiveness during the first exposure and low effectiveness after repeated exposures? Changes in the effectiveness of insecticides are due to mechanisms of tolerance/resistance. These mechanisms include behavioral, molecular, and genetic mechanisms.

CHAPTER 2
MECHANISMS AND DETECTION OF RESISTANCE

Behavioral Mechanisms

The involvement of behavior in the formation of insecticide resistance in mosquito species is often overlooked. Changes in the behavior of targeted species can, however, cause drastic differences in the effectiveness of a given insecticide and its application. Behavioral resistances can be found often in *Anopheles* spp. mosquitoes.

Anopheles mosquitoes tend to rest indoors when not feeding at their active times of the day (Rozendaal et al. 1989). To control for malaria, indoor surfaces of houses within regions at risk, are often sprayed with a residual insecticide, such as DDT or pyrethroids, in order to limit *Anopheles* species from resting indoors and thus limit transmission (Pates and Curtis 2005). In response to insecticide application, *Anopheles* mosquitoes may change their area of resting preference to be outdoors in a forested area to decrease their exposure to the residue of the insecticide. Also if the insecticide has a high irritancy effect, *Anopheles* species can even adapt to an entirely new feeding style. Insecticides with high irritancies cause mosquitoes to become agitated easily upon physical contact which then makes them land for shorter amounts of time. *Anopheles* species can compensate for this by doing what is known as "bite and run" behavior (Pates and Curtis 2005). An example of this was shown in *Anopheles gambiae sensu strictu* in the Tango region of Tanzania where they adapted by simply taking smaller blood-meals at a time until they were fully engorged (Pates and Curtis 2005).

The use of indoor residual insecticides is of limited use against species in other mosquito genera, such as *Culex* or *Aedes*. The species of these genera generally have entirely different

preferences for resting areas than the *Anopheles* species, which naturally limits their exposure to residual insecticides (Brown and Pal 1971).

Another key behavioral adaptation for *Anopheles gambiae sensu strictu* is change in prime feeding times. This is especially a concern in regions where pyrethroid-treated bed-nets are used with the assumption that the mosquitoes (particularly *Anopheles* spp.) will feed mostly at night while people are sleeping. *Anopheles* spp. will sometimes even change their behavior in such a way to integrate a wider range of active feeding times throughout the day as compensation. This makes the use of bed-nets far less effective. In regions where this is a primary means of mosquito control, such behavioral shifts pose a great concern (Pates and Curtis 2005). This trend of pyrethroid-treated bed nets becoming less effective was also observed for the mosquito species *Culex pipiens quinquefasciatus* Say (Irish et al. 2008). It is possible that these *Culex* mosquitoes are also developing a behavioral mechanism of avoidance similar to that of the *Anopheles* mosquitoes. To counter this, a different insecticide that neither species displays tolerance towards should be used for the bed nets and the bed nets must be kept in good condition with minimal holes (Irish et al. 2008).

Molecular Mechanisms

Biochemical mechanisms of insecticide resistance across mosquito species have been an important focus of research studies. These include mutations that cause target site insensitivities within the mosquito nervous system. This insensitivity can occur due to changes that alter sodium channels, *y*-amino butyric acid (GABA) receptors, and acetyl cholinesterase activity within the central nervous system or it can occur due to an increased metabolic rate of insecticide detoxification (Devonshire and Field 1991, Weill et al. 2003, Shang et al. 2008).

An example of a mutation that would affect insecticide resistance is if the mutation occurred in a gene that was responsible for encoding proteins that detoxify toxins entering the body. Another example is if the mutation occurred in a gene that was responsible for encoding proteins directly targeted for binding by the insecticide (Devonshire and Field 1991, Djogbénou et al. 2009). These mutations can arise due to a single substitution of an amino-acid at the active binding site (Weill et al. 2003, Djogbénou 2009). The former example could cause a deleterious effect in the mosquito making it less capable of detoxifying insecticides and thus more susceptible to it; whereas the latter example would cause a resistance phenotype to the insecticide if the insecticide is unable to bind to its intended target.

Insecticide resistance involving mutations in the receptor of the neurotransmitter GABA have been documented in the mosquito *Aedes aegypti*. These receptors are found within neuromuscular cells and the central nervous system. They function as a part of chloride inhibitor channels which control the passage of chloride ions through the gated channels (Shang et al. 2008). Cyclodiene (i.e. Dieldrin), macrocylic-lactones (i.e. Abamectin), and phenyl-pyrazole (i.e. Fipronil) insecticides all target GABA receptors. Point mutations in GABA subunit receptor genes can lead to a resistance phenotype towards all of these insecticide types. This problem is amplified if more than one of these insecticides is used within the same timeframe, since they have the same modes of action. Selective pressures would be amplified strongly towards individual mosquitoes that possess resistance towards that mode of action (Fonseca-González et al. 2009).

Resistances to insecticides can also occur if the key detoxifying proteins are produced in increasing quantities. Augmented production of these detoxifying proteins can occur due to an increase in gene copy numbers of the corresponding genes (Li et al. 2009). Esterase is an

enzymatic protein within mosquitoes that is known to aid detoxification of hazardous substances entering the mosquito body, such as organophosphates (Weill et. al. 2003). The over-expression of esterase produced by esterase genes is thus an important aspect to consider when analyzing insecticide resistance/tolerance within mosquito populations (Cheikh et al. 2009). Organophosphate insecticides specifically target acetylcholinesterase (ace) genes within mosquitoes. Acetylcholinesterase plays an important role in the mosquito nervous system in that it is involved in catalyzing the hydrolysis of acetylcholine—a neurotransmitter (Djogbénou et al. 2008). Resistance is seen when targeted acetylcholinesterase become insensitive towards the organophosphates or similar acting insecticides (Weill et al. 2003).

Acetylcholinesterase is a "B-esterase" or serine esterase. Organophosphate insecticides specifically inhibit the proper functioning of B-esterases (Aldridge 1993). However, mosquitoes have another group of esterases (collectively known as "A-esterases"). These A-esterases function in hydrolyzing organophosphates, carbamates, permethrin (a pyrethroid insecticide), and carboxylic esters (Aldridge 1993, Flores et al. 2004). In response to increased exposure to insecticides of this nature, A-esterases show an increase in expression levels, thus contributing to a greater efficiency at detoxifying these compounds (Aldridge 1993, Flores et al. 2004).

As shown in Figure 2-1, the acetylcholinesterase-1 gene has an important function in the formation of insecticide resistance in mosquitoes towards the carbamate known as "propoxur" (Weill et. al. 2003, Cheikh et al. 2009). Mosquitoes that were resistant to propoxur had unvaryingly high levels of acetylcholinesterase enzymatic activity despite the exposure to increasing concentrations of the insecticide. Mosquitoes that were susceptible to propoxur displayed lower acetyl cholinesterase activity and higher mortality with exposure to increasingly high concentrations of propoxur. This insensitivity is a result of mutations within the ace-1 gene

causing a shift in its binding site compatibility with the propoxur that must bind a specific target site to be effective (Weill et al. 2003). This mechanism was detected in mosquito species of multiple genera, including *Anopheles*, *Culex*, and *Aedes* spp. Since organophosphates share a similar mode of action as carbamates, over-use of organophosphates could elicit a similar response (Florida Coordinating Council on Mosquito Control 1998).

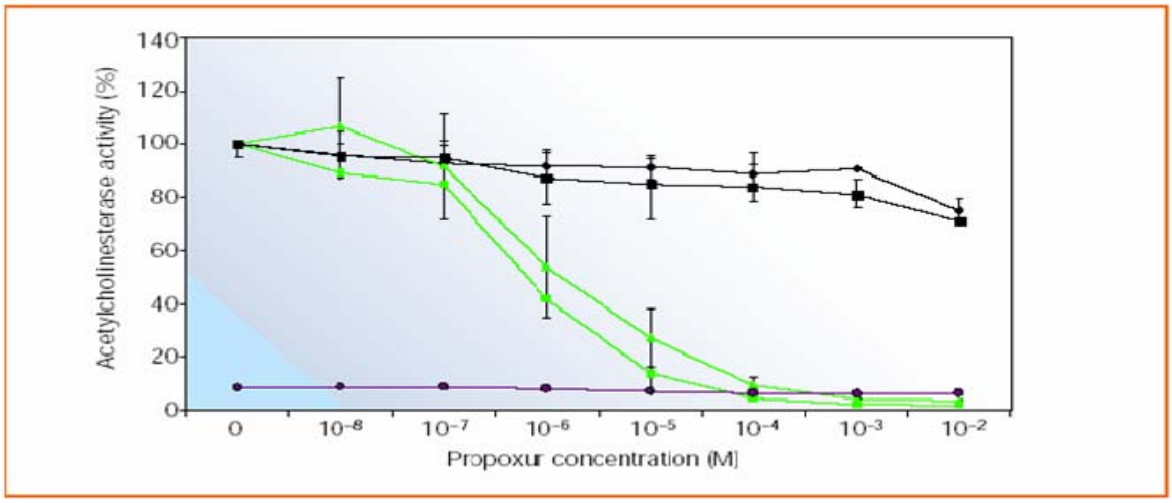

Figure 2-1. Residual acetylcholinesterase activity of susceptible (green) and resistant (black) mosquitoes assayed in homogenates and lysates from transfected S2 cells in the presence of increasing concentrations of the carbamate insecticide Propoxur (Weill et al. 2003).

Genetic Mechanisms

Genetics also plays an important role in the formation and maintenance of insecticide resistance within mosquito populations (Perera 2008). As long as the trait resulting in resistance is heritable, it can be passed on to future generations of mosquitoes, causing the insecticide to become less effective (Apperson and Georghiou 1975).

Examples of inherited genes resulting in resistance towards the insecticides dieldrin and DDT were shown in detailed studies of *Anopheles gambiae* Giles, *Anopheles sundaicus* (Rodenwaldt), *Anopheles albimanus* Wiedemann, *Anopheles quadrimaculatus* Say, and *Culex fatigans* Weidemann mosquito populations (Davidson and Jackson 1961). It was determined that

dieldrin-resistance in all of these species relied on a single heritable genetic factor. This factor was recessive in nature within the *An. sundaicus* populations, but was partially dominant in nature in mosquitoes from populations of the remaining species. It was also determined that the mosquitoes in *Cx. fatigans* populations had a naturally higher resistance towards exposure to DDT than either of the *Anopheles* species despite the same selective pressures. On the other hand, the *Cx. fatigans* populations were much more susceptible towards exposure to dieldrin than either of the *Anopheles* species. Through the use of finely controlled studies of selection of susceptible, hybrid, and resistant strains of mosquitoes, Davidson and Jackson (1961) were able to conclude that resistance towards DDT and dieldrin were independent of one another. This suggests that resistances due to genetic mechanisms are quite diverse in nature (Perera 2008). If the selective pressures from insecticide exposure remain high, insecticide resistance would be maintained within populations (Davidson and Zahar 1973). This would be expected to occur with repeated exposure to the same insecticide over long periods of time. Similarly, insecticide resistance would decline as selective pressures decrease. Such a decline would be the consequence of terminating applications of insecticides in a given region or shifting applications to usage of a different class of insecticide.

Detections of Resistance in Adult Mosquitoes

Resistances can sometimes be difficult to detect. Some strategies include using a combination of several different measurements—including mortality rates, the "knockdown effect", and "irritancy tests" (Hougard 2003). These measurements can be taken using the Bottle Bioassay (McAllister and Brogdon 1999) or the standard World Health Organization kit (WHO 1979) methods of analyzing insecticide resistance. The Bottle Bioassay involves the introduction of a pre-determined number of mosquitoes into glass bottles coated with a known

concentration of insecticide and comparing mortality rates over increments of time (McAllister and Brogdon 1999). Since it is difficult to determine if affected mosquitoes are dead or just immobilized, mortality can be difficult to accurately record. Hence the Bottle Bioassay is useful in testing for the "knock-down effect". The "knock-down" effect involves taking measurements of the amount of time that is required for mosquitoes to recover from the initial shock of landing on an insecticide-treated surface. The WHO kit involves exposing mosquitoes to papers saturated with a known concentration of insecticide (WHO 1979). This test is beneficial in testing for either the "knock-down effect" or the "irritancy effect". The "irritancy effect" involves taking measurements of the amount of time that is required for a mosquito to resume flying around again after it made its "first landing" onto a surface treated with insecticides following a set habituation time period (Guessan 2007). If the time measured during each of these tests has consistently low values then it would be concluded that resistance had developed. Further analysis through the use of molecular techniques could potentially establish the cause of resistance, assuming it is not simply a behavioral adaptation.

CHAPTER 3
ESTERASE ISOLATION AND EXPRESSION IN *CULEX NIGRIPALPUS* Theobald (DIPTERA: CULICIDAE) OF MANATEE COUNTY, FL

Introduction

Esterase is an enzymatic protein in mosquitoes that functions in chemical defense against insecticides (Weill et al. 2003). The objective of this study was to isolate esterase genes from *Culex nigripalpus* Theobald and characterize gene expression in field-collected mosquitoes from Manatee County, Florida. Manatee County has a long history of insecticide usage (http://www.manateemosquito.com/History.html); therefore, there is reason to suspect insecticide resistance to be a concern in this region. This study seeks to better define which insecticide resistance/tolerance mechanisms are used by mosquitoes of Manatee County, thus increasing understanding of insecticide tolerance/resistance status in Florida mosquitoes and enabling the adoption of more effective mosquito control efforts.

Materials and Methods

In collaboration with Manatee County Mosquito Control District (MCMCD), adult female *Cx. nigripalpus* mosquitoes (ca. 300-500) were collected from an area within the MCMCD treatment zones using Centers for Disease Control miniature light traps baited with 3 lb of dry ice. Mosquitoes were chilled and identified. While anesthetized, whole mosquito bodies, body tissues (midguts), and segments (head, abdomen, thorax) were collected using a sterile razorblade and frozen at -80 ^{o}C until use. Female *Cx. nigripalpus* (generation F105) maintained as a colony at the Florida Medical Entomology Laboratory (FMEL) were also used in this study.

Genomic DNA was isolated from whole adult bodies of field-caught *Cx. nigripalpus* females from Manatee County using the Wizard Genomic DNA Purification Kit (Promega,

Madison WI) according to the manufacturer's protocol with the following modifications accounting for extraction from smaller samples including an added incubation period in the initial step and minor changes in the volumes of some reagents: Twelve adult mosquito bodies were homogenized manually in 600 microliters (ul) of Nuclei Lysis Solution and incubated in a 65°C heating block. RNase solution (3 ul) was then added, after which, the tube of homogenized mosquitoes was incubated again at 37º C. Following this incubation, protein precipitation solution (200 ul) was added. The tubes were vortexed and chilled on ice. The tubes were then centrifuged at 13,000 times gravity (xg) and the supernatant collected. Isopropanol (600 ul) was then added and mixed in by inversion. The tubes were centrifuged again at 13,000 xg. The supernatant was carefully removed and the pellet was then washed with 600 ul of 70% ethanol. The pellet was allowed to air dry. The genomic DNA pellet was re-suspended in 500 ul of DNA Rehydration solution and then incubated at 65ºC for 1 hr. This was stored at 4ºC until use.

To purify the DNA, a mixture of Phenol: Chloroform: Isoamyl Alcohol (25: 24: 1) was added to the re-hydrated DNA. The tubes were vortexed and then centrifuged at 13,200 xg at room temperature. The top phase was then collected. Then 0.1x total volume of 2.7M Sodium Acetate of pH 5.2 was added, followed by 2x the total volume of ice cold 100% ethanol. This was mixed by vortexing and incubated at -80ºC and then centrifuged 13,200 xg at room temperature. The pellet was washed using room temperature 70% ethanol and allowed to air dry. The pellet was re-suspended in autoclaved MilliQ water (water that had been filtered through the Millipore, Billerica, MA, system) and incubated overnight at 4ºC.

The extracted DNA was then analyzed by a SmartSpec Plus spectrophotometer (BioRad, Hercules, CA) and on a 1% agarose gel to determine the concentration and to check for degradation.

A polymerase chain reaction (PCR) analysis of the genomic DNA was performed using primers that had been designed based on the sequence of the esterase gene found in *Culex pipiens pipiens* L. The PCR analyses were carried out using the REDTaq Genomic DNA Polymerase kit (Sigma-Aldrich, St. Louis, MO) in a Rapid Cycler™ (Idaho Technology, Inc., Lake City, Utah). The PCR reactions were then analyzed on a 1% agarose gel. Each distinct fragment was extracted from the gel using the QIAgen QIAquick gel extraction kit (QIAgen, Valencia, CA) and the concentration of the PCR fragment was calculated using a SmartSpec Plus spectrophotometer.

The primers used for this study were the following: Beta Forward: 5'-CGA-TCA-TCA-TGA-TGC-GGT-AG-3'; Beta Reverse: 5'-CCA-GAA-GAT-CGT-CGG-CTG-CG-3'; CP3 Forward: 5'-ATT-GGA-AGT-GAG-GAC-AGC-TTG-CAC-3'; CP3 Reverse: 5'-ACC-GTA-CAT-CTC-CAC-TCC-ACT-AGA-3'; Actin-3 (Forward): 5'-CTG-GAT-TCC-GGA-GAT-GGT-GT-3'; Actin-4 (Reverse): 5'-TAG-ACG-GGG-CAA-GGG-CGG-TGA-TTT-3'

Total RNA was extracted from field collected mosquitoes using the TRIzol® Reagent (Invitrogen™, Carlsbad CA). Whole adult female *Cx. nigripalpus* mosquito bodies (12 mosquitoes per tube), body tissues (midguts; 15 per tube), and segments (head, abdomen, thorax; 10 of each per tube) were homogenized in 1000 ul of TRIzol Reagent and the manufacturer's protocol was followed with a modification: Homogenized samples were allowed to incubate at room temperature for 10 minutes and then centrifuged at 4°C at 12,000 xg. The supernatant was collected and 200 ul of chloroform was added. The tubes were then vortexed for 15 seconds. The samples were incubated at room temperature; after which they were centrifuged at 4ºC at 12,000 xg. After this step, the manufacturer's protocol included with the TRIzol reagent was followed. The RNA pellets were then re-suspended in 50 ul of diethylpyrocarbonate-treated

water and incubated at 60ºC. All RNA was stored at -80ºC until ready for use. The concentration of the RNA samples was analyzed using a SmartSpec Plus spectrophotometer. The integrity of the RNA was determined by running it on a 1% agarose/formaldehyde gel and by viewing the gel using an INGenius Gel Documentation System (Syngene Bio Imaging, Frederick, MD) and a Gene Gnome (Syngene Bio Imaging, Frederick, MD).

All reverse transcriptase-PCR (RT-PCR) analyses of the RNA were performed using the same primers as previously described, including an additional primer set: CP4 Forward: 5'-AGT-AAG-CTG-CTG-AAC-AAA-AT-3' and CP4 Reverse: 5'-GTG-GTA-GTG-GAC-GGA-ACA-3'. The RT-PCR analyses were carried out using the protocol included in the Enhanced Avian HS RT-PCR kit (Sigma Aldrich, St. Louis, MO) in a RapidCycler Idaho Technology System (Idaho Technology Inc, Salt Lake City, UT). The RT-PCR reactions were analyzed on a 1% agarose gel. Each distinct fragment was extracted from the gel using the QIAgen QIAquick gel extraction kit and analyzed individually using the SmartSpec Plus spectrophotometer.

All PCR fragments of interest were cloned into the pCR®2.1 cloning vector following the manufacturer's protocol using reagents in the TA Cloning® kit (Invitrogen™, Carlsbad, CA). Recombinant clones were then transformed into bacteria and allowed to grow overnight on kanamycin agar plates. Bacterial colonies that had successfully incorporated the cloning vector containing the gene of interest appeared as white colonies, while those that were non-recombinant were blue. Positive clones were grown overnight at 37°C under agitation in liquid culture containing kanamycin. The plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (QIAgen, Valencia, CA). All positive clones were sequenced using a Beckman Coulter CEQ 8000 Genetic Analysis System with reagents included in the CEQ DTCS Quick Start Kit (Beckman Coulter Inc., Fullerton, CA) and following the manufacturer's protocol. Clones of

interest were sequenced in both directions. Sequence analyses were performed using the Lasergene DNA and protein analysis software from DNASTAR, Inc. (Madison, WI).

Results and Discussion

Esterases are thought to be conserved in mosquito species (Vaughan and Hemingway 1995). Since esterases function in the formation of insecticide resistance, we designed our primers based on known esterase sequences implicated in the resistance phenotype found in the *Cx. pipiens* complex. Through PCR on genomic DNA isolated from whole bodies of female *Cx. nigripalpus*, we isolated a 200 base-pair (bp) fragment using the CP3 primers and a 900 bp fragment using the Beta primers (Fig. 3-1). GenBank database searches (blastn and tblastx) using the sequence of the fragment amplified with the CP3 primers revealed homology at the nucleotide level to an est-3 gene found in *Cx. p. pipiens* (89%; Accession # AJ302090) and at the translation level to a *Cx. p. pipiens* partial est-3 gene encoding esterase-3 (83%; Accession # AM949567). The PCR fragments amplified with the Beta primers matched a translation product of unknown function from *Cx. p. quinquefasciatus*, and its characterization will not be discussed here.

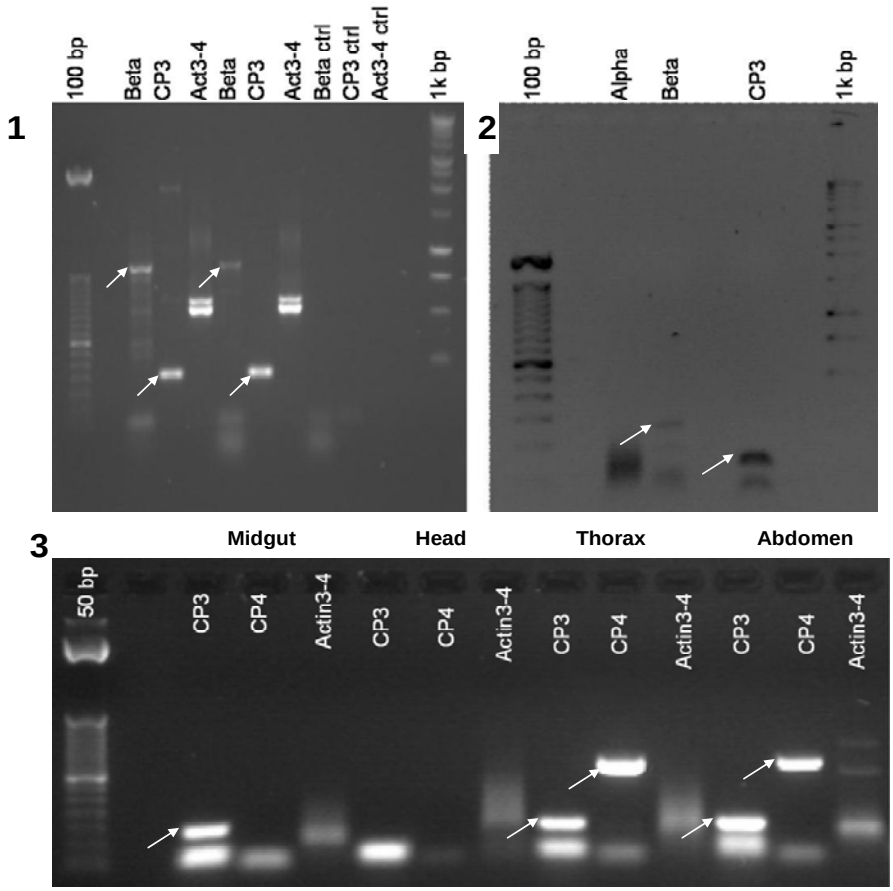

Figure 3-1. **1.)** PCR of whole bodies of *Culex nigripalpus* (Manatee County, Florida). **2.)** RT-PCR of whole bodies of *Cx. nigripalpus* (Manatee County, Florida). **3.)** Expression analysis of colony female *Cx. nigripalpus* (Arrows indicate positive amplification)

Additional primers (CP4) were designed based on the *Cx. p. pipiens* est-3 sequence and were used, along with the CP3 primers, to isolate cDNAs encoding esterase from female *Cx. nigripalpus* whole-body and body segment RNA. Distinct bands were amplified as a 200 bp fragment using the CP3 primers and a 397 bp fragment using the CP4 primers (Fig. 3-1). Sequence analyses were performed on both fragments; however, since the sequence of the 397 bp fragment had a higher homology with esterase proteins, all subsequent sequence comparisons were done using the larger fragment. Expression analyses were performed using both primer sets.

In order to determine the spatial expression of the *Cx. nigripalpus* esterase and to infer a possible function, RT-PCR was performed on RNA isolated from female midgut tissues, heads, thoraces, and abdomens using CP3 and CP4 primers. We found that products from both primers were amplified in RNA from midgut tissue, thorax, and abdomen, but not in heads (Fig. 3-1). Due to the high prevalence of esterase in the abdomen tissues, we expected to detect it in midgut tissues and warrants further expression analysis of the esterase in midgut tissue. Expression of *Cx. nigripalpus* esterase in the abdomen, which includes the midgut, suggests that it has a role in digestion or reproduction (Campbell et al. 2003, Lima-Catelani et al. 2004). The esterase expression found in the thorax may be attributed to the presence of salivary glands which have been shown to secrete esterases used to aide digestion and defenses during feeding (Argentine and James 1995, Calvo and Ribeiro 2006).

The 397 bp fragment, CN Temsha est-1 (Accession # GO343531), encodes a putative translation product of 131 amino acids, and is incomplete at the 5'- and 3'-ends (CN TEMSHA EST-1; Fig. 3-2). The *Cx. nigripalpus* esterase-like translation product displayed high homology with a number of mosquito gene translation products suggested to play a role in insecticide resistance, including *Cx. p. pipiens* esterase A5 (96%, Accession # AY545983; Buss and Callaghan, 2004), *Cx. p. quinquefasciatus* estalpha2 esterase (96%, Accession # Z47988, Vaughan and Hemigway 1995), and with *Aedes aegypti* L. alpha-esterase partial mRNA (85%, Accession # XM_001654459; Nene et al. 2007). The alignment of the CN Temsha est-1 putative translation product with esterase proteins from other mosquitoes is shown (Fig. 3-2; Buss and Callaghan 2004, Cui et al. 2007, Nene et al. 2007, Vaughan et al. 1997). The high homology suggests that it is a member of the esterase family, which indicates that *Cx. nigripalpus*

mosquitoes from Manatee County, FL express an esterase whose functions would include digestion in blood-feeding or insecticide detoxification.

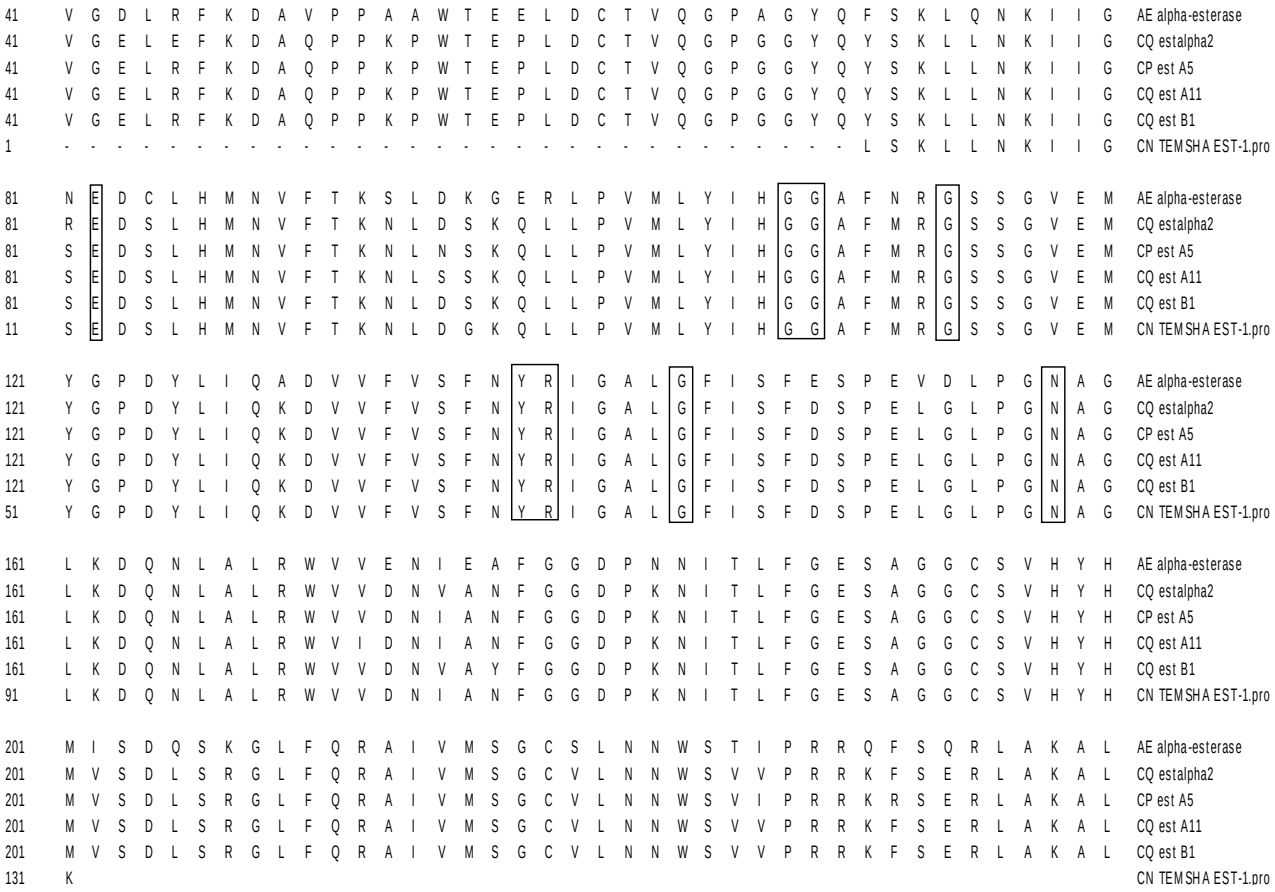

Figure 3-2. Protein alignment of the putative translation product of CN TEMSHA EST-1 with homologous esterase proteins. (The boxed amino acids point out conserved residues among esterase proteins; the numbers represent amino acid numbers.)

Esterases have many functions in the mosquito; therefore it is important that we determine whether the esterase isolated from *Cx. nigripalpus* plays a role in insecticide tolerance/resistance (Lima-Catelani *et al.* 2004, Flores et al. 2004). The results obtained in this study laid the foundation for further studies used to define the involvement of this gene in insecticide tolerance/resistance in *Cx. nigripalpus* from Manatee County, FL and provide much needed information in the district's operational control efforts.

CHAPTER 4
ESTERASE EXPRESSION AND POPULATION ANALYSES OF FEMALE *CULEX NIGRIPALPUS* Theobald (DIPTERA: CULICIDAE) OF MANATEE COUNTY, FL

Introduction

In previous experiments, a cDNA fragment (Temsha est-1; Accession # GO343531) was isolated from female *Cx. nigripalpus* of Manatee County, FL translation product shared a high homology with alpha esterase encoding genes from mosquitoes. In the experiment described herein, our goals were the following: to obtain a larger cDNA clone encoding *Cx. nigripalpus* esterase Temsha est-1 (TE-1), to determine when and where TE-1 is expressed in female *Cx. nigripalpus* body segments and tissues, and to analyze its expression levels across individual mosquitoes from distinct populations in Manatee County Florida. These studies help to determine the function of TE-1 and serve as preliminary information in verifying the role of *Cx. nigripalpus* TE-1 in the formation of insecticide resistance in Manatee County, Florida *Cx. nigripalpus*.

Materials and Methods

In collaboration with Manatee County Mosquito Control District (MCMCD), adult female *Culex nigripalpus* field mosquitoes (ca. 300-500) were collected and used in this study. Female *Cx. nigripalpus* were maintained as described previously. Mosquitoes were chilled and identified. While anesthetized, whole mosquito bodies, body tissues (midguts), and segments (head, abdomen, thorax) were collected using a sterile razorblade and frozen at -80 ^{o}C until use. *Culex nigripalpus* used in this study were collected from the following geographic locales: "G2" (Manatee County; Fig. 4-1), "G4" (Manatee County; Fig. 4-1), and the FMEL ("Colony" F105 and "Hybrids" F28). The "Hybrids" (F28) represent "Colony" *Cx. nigripalpus* that had been out-crossed with wild *Cx. nigripalpus* and maintained over 28 generations. The "G2" area is located

near a salt marsh zone in Manatee County; whereas the "G4" area is located further inland near an agricultural zone. "G2" and "G4" are 5 miles apart. Any mosquitoes not immediately used were kept frozen at -80°C.

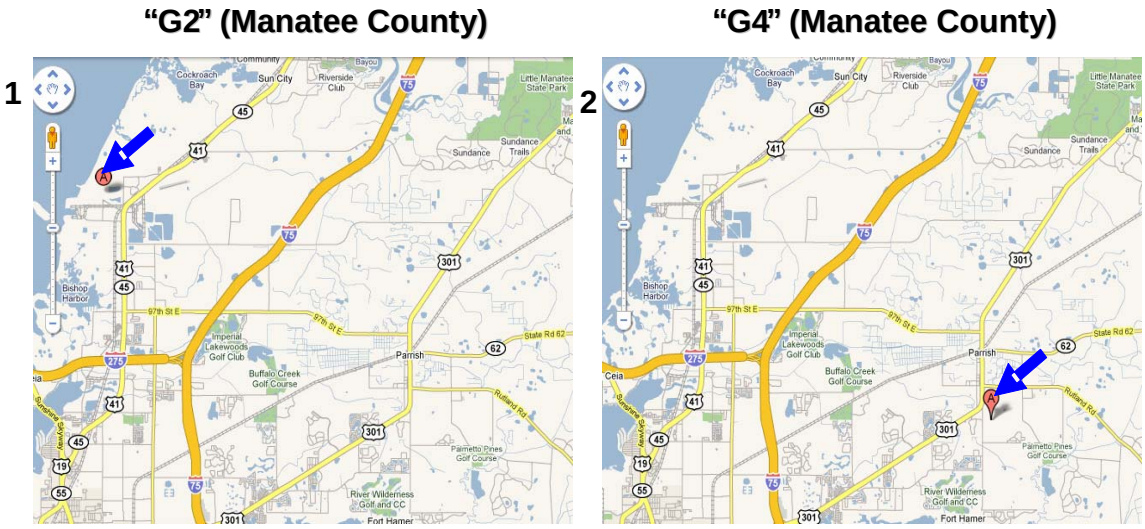

Figure 4-1. **1.)** Map of "G2" (Manatee County) location: Piney Point Road, Palmetto, FL 34221 **2.)** Map of "G4" (Manatee County) location: Britt Road, Parrish, FL 34219. (Locations indicated by arrows.)

Tissues and body segments were dissected from the long standing *Cx. nigripalpus* colony "Hybrids" (F28) before and after blood-feeding as described elsewhere (Smartt and Erickson 2008). Non-blood-fed and blood-fed adult female colony *Cx. nigripalpus* were anesthetized with exposure to cold and tissues of interest dissected. Body segments (head, thorax, abdomen; 10 per tube) and body tissues (midguts, ovaries; 20 per tube) were collected and stored at -80C until use. The end of this feeding period was termed the "0 hour (h)". Body segments and body tissues were collected from the blood-fed individuals at each of the following time points: 0 h, 3 h, 6 h, 9 h, 12 h, 24 h, 48 h, and 72 h after blood-feeding.

The primers used for this study were the following: CP3 Forward: 5'-ATT-GGA-AGT-GAG-GAC-AGC-TTG-CAC-3'; CP3 Reverse: 5'-ACC-GTA-CAT-CTC-CAC-TCC-ACT-AGA-3'; CP4 Forward: 5'-AGT-AAG-CTG-CTG-AAC-AAA-AT-3'; and CP4 Reverse: 5'-

GTG-GTA-GTG-GAC-GGA-ACA-3; Actin-3 (Forward): 5'-CTG-GAT-TCC-GGA-GAT-GGT-GT-3'; Actin-4 (Reverse): 5'-TAG-ACG-GGG-CAA-GGG-CGG-TGA-TTT-3'.

Total RNA was extracted from non-blood-fed and blood-fed field collected and colony mosquitoes using the TRIzol® Reagent (Invitrogen™, Carlsbad CA). Whole adult female *Cx. nigripalpus* mosquito bodies (12 mosquitoes per tube), body tissues (midguts; 15 per tube), and segments (head, abdomen, thorax; 10 per tube) were homogenized in 1000 ul of TRIzol Reagent and the manufacturer's protocol was followed as described previously in Chapter 3. The RNA pellets were then re-suspended in 50 ul of diethylpyrocarbonate-treated water and incubated at 60ºC. All RNA was stored at -80ºC until ready for use. The concentration of the RNA samples was analyzed using a SmartSpec Plus spectrophotometer. The integrity of the RNA was determined by running it on a 1% agarose/formaldehyde gel and by viewing the gel using an INGenius Gel Documentation System (Syngene Bio Imaging, Frederick, MD).

All reverse transcriptase-PCR (RT-PCR) analyses of the RNA were performed using the primers as previously described. The RT-PCR analyses were carried out using the protocol included in the Enhanced Avian HS RT-PCR kit (Sigma Aldrich, St. Louis, MO) in a RapidCycler Idaho Technology System (Idaho Technology Inc, Salt Lake City, UT). The RT-PCR reactions were analyzed on a 1% agarose gel.

All PCR fragments of interest were cloned and recombinant clones were selected as previously described in Chapter 3. The plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (QIAgen, Valencia, CA) and sequenced using a Beckman Coulter CEQ 8000 Genetic Analysis System with reagents included in the CEQ DTCS Quick Start Kit (Beckman

Coulter Inc., Fullerton, CA) and following the manufacturer's protocol. Sequence analyses were performed using the Lasergene DNA and protein analysis software from DNASTAR, Inc. (Madison, WI).

For population analyses, individual adult female *Cx. nigripalpus* were separated by locality (G2, G4, and Colony). RNA was extracted from each individual mosquito and RT-PCR was performed on the extracted RNA using the CP4 esterase primers as described in Chapter 3. The RT-PCR reactions were analyzed on a 1%-agarose gel to compare differences in esterase expression between the populations.

Results and Discussion

Esterases have many potential functions in the mosquito (Lima-Catelani et al. 2004, Flores et al. 2004); therefore it is important that we determine whether our Cx. nigripalpus esterase TE-1 has a role in insecticide tolerance/resistance in the *Cx. nigripalpus* of Manatee County, FL or if its role involves digestion as we have shown by expression in midgut tissue (Eans et al. 2009a unpublished). Using the CP4 esterase primers for TE-1, expression in female *Cx. nigripalpus* ("Hybrids" F28) body segments and tissues was analyzed before and after blood-feeding. TE-1 was highly expressed in the head, thorax, abdomen, and midgut tissues in non-blood-fed mosquitoes and in the early hours after feeding in the blood-fed mosquitoes. In the thorax and midgut tissues, there was a clear decline in expression over time (Fig. 4-2). This supports the conclusion from previous experiments that TE-1 may be involved in feeding and digestion in female *Cx. nigripalpus* (Eans et al. 2009b unpublished). Esterases are secreted through the proboscis in response to both feeding on blood meals and sugar meals, therefore its presence in the head could be due to the presence of enzymes released by the salivary glands (Argentine and James 1995).

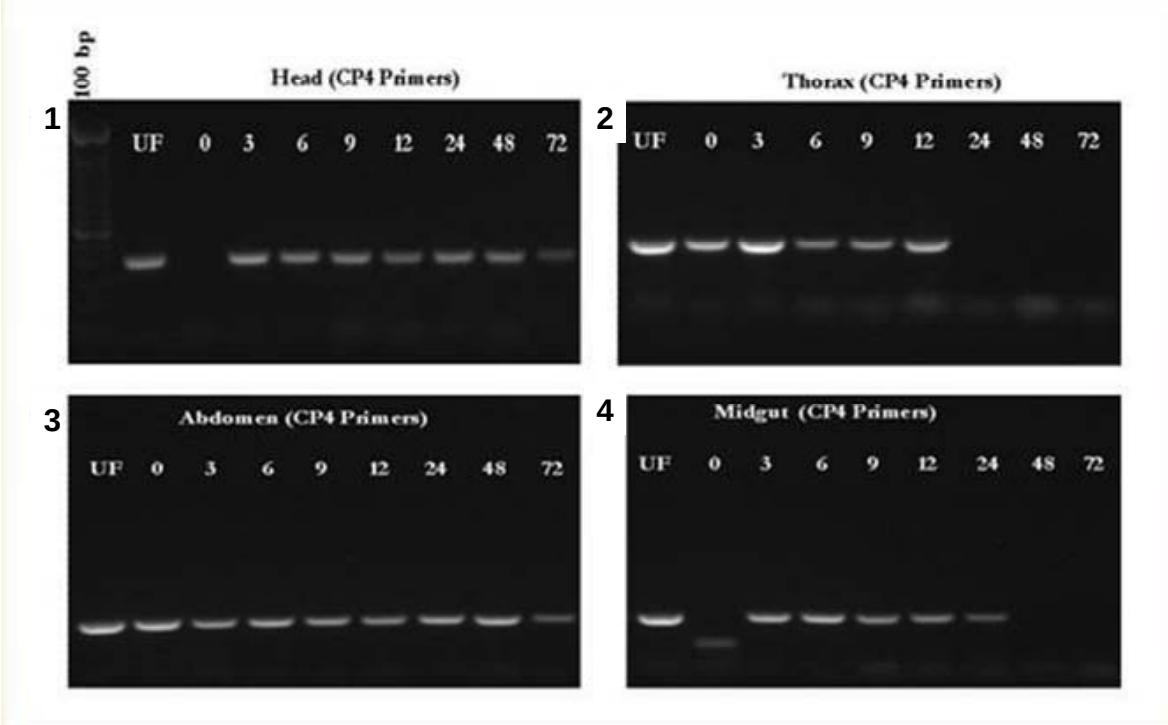

Figure 4-2. Amplification of TE-1 in RNA extracted from heads, thoraces, abdomens, and midguts dissected from unfed (UF) female *Cx. nigripalpus* and blood-fed female *Cx. nigripalpus* over a 72-hour time period (0, 3, 6, 9, 12, 24, 48, and 72 hours after being blood-fed).

The expression of TE-1 was characterized in unfed individual female *Cx. nigripalpus* from the following locations for population analyses: "G2" (Manatee County), "G4" (Manatee County), and "Colony" (FMEL). There was a distinct difference in the levels of expression of TE-1 in individuals, depending on the location in which they were collected. The majority of the individuals taken from the "G2" area of Manatee County were positive for the expression of TE-1; however, the majority of individuals from the "G4" area of Manatee County had very little expression of TE-1 (Fig.4-3). Individuals from the "Colony" of FMEL had mixed results between those that were positive for expression of TE-1 and those that had little expression of TE-1 (Fig. 4-3).

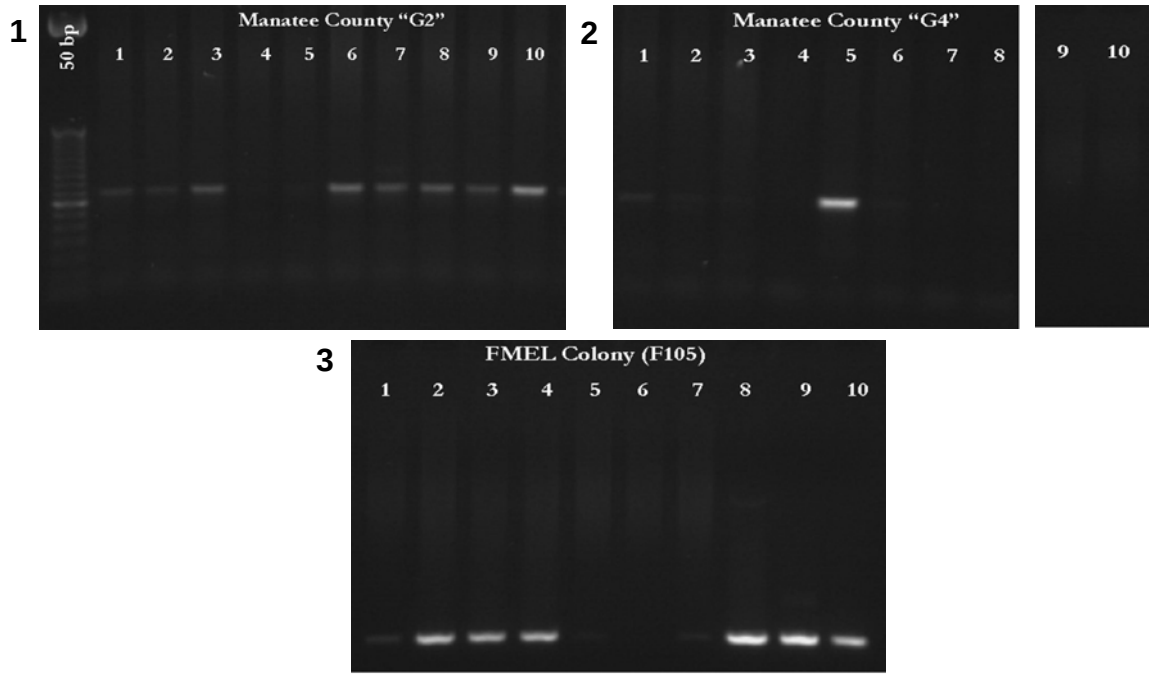

Figure 4-3. Amplification TE-1 using RNA extracted from individual female *Cx. nigripalpus* trapped from "G2" (Manatee County)**,** "G4" (Manatee County) and the FMEL Colony (F105).

Since esterases are known to be involved in insecticide tolerance/resistance formation in mosquitoes, these results may indicate a difference in tolerance/resistance within these populations of *Cx. nigripalpus* due to the distinct variation in TE-1 expression between the locations (Flores et al. 2004). Future studies are needed to compare differences in tolerance between the *Cx. nigripalpus* from each area through the use of the Bottle Bioassay. Individuals with high susceptibility towards a given insecticide and those that are tolerant towards the insecticide need to be compared for differences in TE-1 expression between the most and least susceptible individuals. Based on earlier findings from other researchers, if TE-1 is involved in insecticide resistance, one would expect that high levels of TE-1 would be expressed at higher levels in the tolerant individuals and lower expression in the susceptible individuals (Flores et al. 2004, Cui et al. 2007).

The results of these experiments further enhance our understanding for the role TE-1 has in *Cx. nigripalpus.* Future studies are needed to amplify the complete sequence for TE-1 using the esterase primers. We will also repeat all expression studies of TE-1 amplification in RNA extracted from heads, thoraces, abdomens, and midguts of unfed and fed individuals to make certain all results remain consistent. As mentioned previously, we will continue our population analyses by comparing expression levels of TE-1 in tolerant and susceptible individuals. If there is a correlation between the expression of TE-1 and resistance, then one could potentially develop an assay using TE-1 as an indicator for development of resistance in populations of *Cx. nigripalpus*. This would greatly enhance mosquito control efforts in mosquito control district's like the Manatee County Mosquito Control District.

CHAPTER 5
PROBLEMS WITH CHEMICAL CONTROL

In order to become more effective at controlling mosquito populations, it is imperative to have a greater understanding of the mechanisms involved in insecticide resistance within different mosquito populations. Molecular, genetic, and behavioral factors involved in resistance/tolerance in mosquitoes all play significant roles in why insecticides become less effective with time (Hemingway and Ranson 2000). If we only looked at one factor at a time it could create misinterpretations if the results from analyzing one particular factor conflicted with the results obtained from analyzing another factor. This could lead to false conclusions. Likewise, the mosquitoes from a given population could be using a combination of these factors, meaning that one would need to account for multiple mechanisms involved in order to maintain effective control (Corbel et al. 2007).

For example, studies may show that there is little change in the biochemistry or genetics from one generation to the next in *Anopheles* mosquitoes, which suggests that these mosquitoes would still be susceptible to pyrethroid-treated bed-nets and indoor treatments of residual insecticides; however, control measures might show that their populations are not declining after repeated treatments (Corbel et al. 2007). Without understanding the behavior of the targeted mosquito as well, one might not know how to interpret these rather confusing results. By understanding all aspects of resistance, one could perhaps conclude that a change in behavior might be causing the numbers of this population of mosquitoes to remain constant (Pates and Curtis 2005). This could be verified by experimentation to determine if these *Anopheles* are simply limiting the contact they have with treated surfaces or adopting different feeding and resting habits (Florida Coordinating Council on Mosquito Control 1998).

It is also important to understand how each insecticide functions and any potential impacts on the surrounding environment. The goal is to minimize the rate of resistance forming in response to repeated applications of a given insecticide (Florida Coordinating Council on Mosquito Control 1998). One could achieve this is by doing one of the following: using the lowest concentration of an insecticide required for maintaining effective control on a given mosquito population, reducing the frequency of applications, and switching between different classes of insecticides. For example, cyclodiene (i.e. Dieldrin) and phenyl-pyrazole (i.e. Fipronil) insecticides both use the same mode of action to decimate their intended target—by disrupting the function of GABA receptors for mosquito neurotransmitters (Shang et al. 2008). If the targeted population of mosquitoes started to form a resistance towards applications of cyclodiene, then switching to the use of phenyl-pyrazole would show a decreased effectiveness as well (Shang et al. 2008). Applying both of these insecticides in the same region during the same time period would only speed up the formation of resistance towards both compounds until neither one was effective. To slow down tolerance one could do one of the following: use low dosages thus allowing for susceptible individuals to continue passing along their alleles throughout the next generations, apply the insecticides at low frequencies, use insecticides that have shorter persistence in the environment, use fast-release formulations, and treat only the areas that require control (Florida Coordinating Council on Mosquito Control 1998).

These problems are only augmented by the fact that there is a limit on the dose that can be applied before the toxicity of the insecticides becomes too harmful for people and the surrounding environment. As long as the use of insecticides remains the main form of mosquito control, the formation of resistant mosquito populations will always pose a problem (Hemingway and Ranson 2000). There exists a never-ending race to evolve novel insecticides and maintain

control over mosquito populations, but it is a race that can be easily lost if caution is not taken (Li et al. 2009). This is why it is important to minimize the use of insecticides when possible (Becker et al. 2003).

Another issue with using insecticides for mosquito control is that detection of resistance development in a mosquito population can often be difficult to judge accurately (Hougard et al. 2003). Judging mosquito mortality, for instance, can be subject to personal interpretation. Many insecticides have a short-term knock-down effect that causes the mosquito to be incapable of moving as a result of the initial distress on its nervous system. This paralysis can last over a wide range of time, from as short as a few seconds to as long as a few days (Hougard et. al. 2003).

Different insecticides can also have different levels of efficiency depending on the climate in which it is used. Temperature and humidity both play large roles in an insecticide's relative toxicity (Hodjati and Curtis 1999). In order to test how efficient a given insecticide will be one must always keep in mind the environment of targeted mosquito populations. To account for the potential effects of varying climates, tests comparing the relative effectiveness of insecticides for use at a given location would ideally be performed under the same conditions. One could achieve this by performing the tests in controlled laboratory settings (Hougard et al. 2003).

CHAPTER 6
ALTERNATIVES TO INSECTICIDE USAGE

There are many alternatives to controlling mosquito populations and reducing the chance that one will be bitten by mosquitoes that do not require the use of insecticides. Minimizing the use of insecticides helps to slow down the formation of tolerance in mosquito populations (Florida Coordinating Council on Mosquito Control 1998). Some alternatives include minimizing the number of standing water sources available as breeding sites for mosquitoes, minimizing one's contact with mosquitoes, and using biological control measures.

A large factor in controlling mosquito populations is to limit the number of breeding sites to which they have access (Florida Coordinating Council on Mosquito Control 1998). The fewer sites available to them, the smaller their population becomes as a result. This can be done in several ways. One way is to discard all unnecessary sources of water collecting. Water can build up in flower pots, cans, and other discarded containers. Inverting these containers when not in use or simply drilling holes at the bottom of these containers allows excess water to drain out of them. The same is true for outdoor swimming pools, which should be kept drained of water when not in use (Becker et al. 2003). For important bodies of water that cannot be kept drained due to constant usage, such as bird baths, outdoor pet dishes, and fire buckets, the solution is to refill these containers with fresh water at least once every week. Around buildings, water should be kept free-flowing by making sure roof drains and gutters are clear of debris and air conditioning units should be maintained to prevent improper build-up of fluids due to condensation (Becker et al. 2003). In agricultural systems, farmers are advised to install irrigation and water channels in such a way that they do not form mosquito breeding sites (Becker et al. 2003). City officials can also contribute to limiting the amount of breeding sites available to mosquitoes by ensuring roads, drains, storm water areas, sewage and street waste

systems flow properly. Also they can help reduce this by minimizing potential mosquito breeding sites from public areas and making sure that waste water plants are properly managed (Becker et al. 2003). The use of organic surface films on standing water systems, such as waste water plants, can help to suffocate mosquito larvae and newly emerged adults. Decreasing the survival rate of mosquito larvae results in fewer adults that will emerge later (Kady et al. 2008).

Reducing the amount of contact one has with mosquitoes while outdoors is also important. This limits the chances of coming in contact with infected mosquitoes. Examples of this would be to wear long pants and shirts with sleeves when feasible while outdoors during peak hours of mosquito activity. It is also advised to avoid sitting outside after dark in locations where mosquitoes are active and to use mosquito netting when sleeping in an unscreened location. Installing mesh screens on windows, doors, and porches can limit the amount of access mosquitoes have to indoor environments (Becker et al. 2003).

The use of biological control is also a useful alternative to insecticide use. Biological control agents include the use of fungi, bacteria, microsporidia, viruses, and natural predators. Insect-pathogenic fungi, such as *Metarhizium anisopliae* and *Beauveria bassiana*, are commercially produced for controlling a wide range of insect pests (Scholte 2005). Fungi function by penetrating through the cuticle of their mosquito hosts and are capable of killing the mosquito with minimal contact. Unlike fungi, bacteria, microporidia, and viruses must first be ingested before they kill their hosts (Scholte 2005). The introduction of natural predators is also beneficial in reducing mosquito populations. In ornamental ponds and other permanent water sources, mosquito fish (*Gambusia* spp.) are often added to the water of common breeding sites to reduce the number of mosquito larvae present (Pates and Curtis 2005). Establishing mosquito populations of the genus *Toxorhynchites* in regions that have problems with controlling mosquito

vector populations is also beneficial. *Toxorhynchites* spp. adults do not blood-feed in their lifetimes; yet they can be useful predators of the larvae of other mosquito species (Jones and Schreiber 1994). Populations of *Toxorhynchites* can be difficult to establish, however, which limits their usage as a biological control agent in certain regions.

The uses of alternative methods of control rather than chemical methods are effective in slowing down the formation of insecticide resistance. Simply denying the mosquitoes a suitable place to lay their eggs (i.e. reducing their access to standing bodies of water) can serve as an effective means of limiting mosquito populations without having to resort to the use of harmful chemicals (Becker et al. 2003). Reducing one's contact with the mosquitoes is also beneficial in controlling the spread of mosquito-vectored diseases. If a biological control agent becomes well established, it can serve as a continuous source of mosquito control (Jones and Schreiber 1994). These alternative methods of control when combined with limited use of insecticides are important for maintaining utmost control over mosquito populations.

CHAPTER 7
CONCLUSION

Due to the rapid nature of insecticide resistance formation in response to repeated exposure to insecticides, we must continuously race to stay one step ahead in order to maintain control of populations of mosquito species throughout the world (Hemingway and Ranson 2000, Coleman and Hemingway 2007, Li et al. 2009). The importance of maintaining control is even more imperative because mosquitoes play a role in spreading pathogenic diseases, such as yellow fever, dengue, filariasis, and encephalitis (Greenwood 2002).

Esterases are known to be involved in the formation of insecticide tolerance/resistance (Flores et al. 2004, Cheikh et al. 2009), therefore the first focus of this study was to isolate an esterase from female *Culex nigripalpus* of Manatee County, FL., The *Cx. nigripalpus* esterase (TEMSHA est-1; Accession # GO343531) had a high homology with *Cx. p. pipiens* esterase A5 (96%, Accession # AY545983; Buss and Callaghan, 2004), *Cx. p. quinquefasciatus* estalpha2 esterase (96%, Accession # Z47988, Vaughan and Hemigway 1995), and with *Ae. aegypti* L. alpha-esterase partial mRNA (85%, Accession # XM_001654459; Nene et al. 2007) esterases previously shown to be important in detoxifying insecticides. Therefore, Temsha est-1 is likely a member of the esterase family of proteins that could potentially detoxify insecticides.

Esterases have many functions in mosquitoes, therefore this study focused on where and when Temsha est-1 (TE-1) was expressed in *Cx. nigripalpus*. The level of expression of TE-1 in RNA extracted from female Cx. nigripalpus body segments (heads, thoraces, and abdomens) and from body tissues (midguts) was determined and it was found that TE-1 was highly expressed in the thorax and abdomen of *Cx. nigripalpus*. The levels of expression of TE-1 in body segments and body tissues dissected before and after blood feeding were determined. TE-1 was highly expressed in thoraces and midgut tissues before and immediately after blood feeding; however,

over time there was a noticeable decrease in TE-1 expression in both the thorax and midgut. This suggests that TE-1 could be involved in feeding and digestion (Argentine and James 1995, Calvo and Ribeiro 2006).

The level of expression of TE-1 in RNA extracted from individuals in three distinct populations of Cx. nigripalpus—"G2" (Manatee County), "G4" (Manatee County), and in a long standing colony (F105) was characterized. Individuals varied in the expression of TE-1 depending on the location they were collected from. Most individuals from the "G2" area showed high expression of TE-1. Most individuals from the "G4" region, on the other hand, showed low expression of TE-1. Our lab "Colony" were mixed with individuals that had high and low expression of TE-1. If TE-1 plays a role in insecticide resistance, we would expect that there exists a difference in tolerance to insecticides between individuals that have high expression of TE-1 and individuals that have low expression of TE-1 (Flores et al. 2004, Cheikh et al. 2009). Further studies are needed to analyze the differences in tolerance levels by using the Bottle Bioassay to determine the effectiveness of exposure to insecticides. The expression of TE-1 would then be compared from RNA extracted from tolerant individuals and from susceptible individuals. Our hypothesis is that high levels of TE-1 would be expressed highest in the tolerant individuals and lowest in the susceptible individuals (Flores et al. 2004, Cui et al. 2007).

If we can use our knowledge of mosquito behavioral, molecular, and genetic mechanisms to better predict potential tolerance/resistance to insecticides, we can develop efficient alternative mosquito control measures. Monitoring changes in insecticide detoxification enzymes, feeding

and resting behavior, mutations of important target-sites, or shifts in resistance allele frequencies throughout each mosquito population can help to serve as early indicators of the development of tolerance/resistance.

REFERENCES CITED

Aldridge WN. 1993. The esterases: Perspectives and problems. *Chem. Biol. Interactions,* 87: 5-13.

Apperson CS, Georghiou GP. 1975. Inheritance of resistance to organophosphorus insecticides in *Culex tarsalis* Coquillet. *Bull. World Health Organ,* 52: 97-100.

Argentine JA, James AA. 1995. Characterization of a salivary gland-specific esterase in the vector mosquito, *Aedes aegypti. Insect Biochem. Mole.c Biol.* 25: 621-630.

Becker N, Petrić D, Boase C, Zgomba M, Lane J, Dahl C, Kaiser AC. 2003. Mosquitoes and their control. Springer. Pp. 407-410.

Brown AWA. 2006. Mechanisms of resistance against insecticides. *Annu. Rev. Entomol.* 5: 301-326.

Brown AWA, Pal R. 1971. Insecticide resistance in arthropods. W.H.O. Monogr. 38.

Buss DS, Callaghan A. 2004. Molecular comparisons of the *Culex pipiens* (L.) complex esterase gene amplicons. *Insect Biochemistry and Molecular Biology* 34: 433-441.

Calvo E, Ribeiro JMC. 2006. A novel secreted endonuclease from *Culex quinquefasciatus* salivary glands. *Journ. Experim. Biol. 209: 2651-2659.*

Campbell PM, Robin GCQ, Court LN, Dorrian SJ, Russell RJ, Oakeshott JG. 2003. Developmental expression and gene/enzyme identifications in the alpha esterase gene cluster of *Drosophila melanogaster*. *Insect Mol. Biol.* 12: 459-471.

Cheikh RB, Berticat C, Berthomieu A, Pasteur N, Cheikh HB, Weill M. 2009. Genes conferring resistance to organophophorus insecticides in *Culex pipiens* (Diptera: Culicidae) from Tunisia. *J. Med. Entomol.* 46: 523-530.

Clements AN. 2000. The biology of mosquitoes: Development, nutrition, and reproduction. *CABI Publishing.* 1: 1-441.

Coleman M, Hemingway J. 2007. Insecticide resistance monitoring and evaluation in disease transmitting mosquitoes. *J. Pestic. Sci.*, 32: 69-76.

Cui F, Weill M, Berthomieu A, Raymond M, Qiao CL. 2007. Characterization of novel esterases in insecticide-resistant mosquitoes. *Insect Biochem. Mol. Bio.,* 37: 1131-1137.

Davidson G, Zahar AR. 1973. The practical implications of resistance of malaria vectors to insecticides. *Bull. Wld. Hlth. Org*. 49: 475-483.

Davidson G, Jackson CE. 1961. Insecticide resistance in mosquitoes. *Nature* 190: 364-365.

Dent D. 1995. Integrated pest management. *Springer,* pp 1-76.

Devonshire A, Field L. 1991. Gene amplification and insecticide resistance. *Annu. Rev. Entomol.* 36: 1-23.

Djogbénou L, Akogbéto M, Chandre F. 2008. Presence of insensitive acetylcholinesterase in wild populations of *Culex pipiens quinquefasciatus* from Benin. *Acta. Tropica*. 107: 272-274.

Eans SE, Frommer RL, Anderson S, Yost S, Smartt CT. 2009a. Esterase expression and population analyses of *Culex nigripalpus* Theobald (Diptera: Culicidae) of Manatee County, FL. [unpublished].

Eans SE, Frommer RL, Smartt CT. 2009b. Esterase isolation and expression in *Culex nigripalpus* Theobald (Diptera: Culicidae) of Manatee County, FL. [unpublished].

Flores AE, Albeldano-Vazquez W, Salas IF, Badii MH, Becerra HL, Garcia GP, Fuentes SL, Brogdon WG, Black WC, Beaty BJ. 2004. Elevated α-esterase levels associated with permethrin tolerance in *Aedes aegypti* (L.) from Baja California, Mexico. *Pesticide Biochemistry & Physiology* 82: 66-78.

Florida Coordinating Council on Mosquito Control. 1998. Florida Mosquito Control: The state of the mission as defined by mosquito controllers, regulators, and environmental managers. University of Florida. {http://fmel.ifas.ufl.edu/white_paper/state.shtml}

Fonseca-González I, Quiñones ML, McAllister J, Brogdon WG. 2009. Mixed-function oxidases and esterases associated with cross-resistance between DDT and lambda-cyhalothrin in *Anopheles darlingi* Root 1926 populations from Colombia. *Memórias do Instituto Oswaldo Cruz*, 104: 18-26.

Greenwood B, Mutabingwa T. 2002. Malaria in 2002. *Nature* 415: 670-672.

Guessan RN, Boko P, Odjo A, Akogbéto M, Yates A, Rowland M. 2007. Chlorfenapyr: A pyrrole insecticide for the control of pyrethroid or DDT resistant *Anopheles gambiae* (Diptera: Culicidae) mosquitoes. *Acta. Tropica.* 102: 69-78.

Hayes EB, Komar N, Nasci RS, Montgomery SP, O'Leary DR, Campbell GL. 2005. Epidemiology and transmission dynamics of West Nile virus disease. *Emerg. Infect. Diseases* 11: 1167-1173.

Hemingway J, Ranson H. 2000. Insecticide resistance in insect vectors of human disease. *Annu. Rev. Entomol*. 45: 371-391.

Hodjati MH, Curtis CF. 1999. Effects of permethrin at different temperatures on pyrethroid-resistant and susceptible strains of *Anopheles*. *Med, Vet. Entomol*. 13: 415-422.

Hougard JM, Duchon S, Darriet F, Zaim M, Rogier C, Guillet P. 2003. Comparative performances, under laboratory conditions, of seven pyrethroid insecticides used for impregnation of mosquito nets. *World Health Org.* 81 (5): 324-333.

Huhn GD, Sejvar JJ, Montgomery SP, Dworkin MS. 2003. West Nile virus in the United States: An update on an emerging infectious disease. *Amer Family Phys.* 68: 653-660.

Irish SR, Guessan RN, Boko PM, Metonnou C, Odjo A, Akobeto M, Rowland M. 2008. Loss of protection with insecticide-treated nets against pyrethroid-resistant *Culex quinquefasciatus* mosquitoes once nets become holed: An experimental hut study. *Parasites & Vectors,* 1: 17.

Jones C, Schreiber E. 1994. The carnivores, *Toxorhynchites*. *Wing Beats*. 5(4): 4.

Kady GA, Kamel NH, Mostey YY, Bahght IM. 2008. Comparative toxicity of two bio-insecticides (spinotoram and vertemic) compared with methomyl against *Culex pipiens* and *Anopheles multicolor*. *World Journ. Agric. Sci*. 4: 198-205.

Kidwell MG, Wattam AR. 1998. An important step forward in the genetic manipulation of mosquito vectors of human disease. *Proc. Natl. Acad. Sci. USA,* 95: 3349-3350.

Kyle JL, Harris E. 2008. Global spread and persistence of Dengue. *Annu. Rev. Microbiol*. 62:71-92.

Levy S. 2007. Mosquito modifications: new approaches to controlling malaria. *BioScience*. 17 (10): 2-7.

Li CX, Dong YD, Song FL, Zhang XL, Gu WD, Zhao TY. 2009. Company amplification of *estα2/estβ2* and correlation between esterase gene copy number and resistance to insecticides in the field *Culex pipiens pallens* strains collected from Beijing, China. *J. Med. Entomol.* 46: 539-545.

Lima-Catelani ARA, Ceron CR, Bicudo HEMC. 2004. Variation of genetic expression during development, revealed by esterase patterns in *Aedes aegypti* (Diptera, Culicidae). *Biochem. Genet.* 42:69-84.

McAllister J, Brogdon W, 1999. The bottle bioassay for measuring resistance. Wing Beats 10: 18-21.

Nayar JK. 1982. Bionomics and physiology of *Culex nigripalpus* (Diptera: Culicidae) of Florida: An important vector of diseases. Gainesville: IFAS. 827. 72 p. Institute of Food and Agricultural Sciences, Univ. of Florida, Gainesville, Florida.

Nene V, Wortman JR, Lawson D, Haas B, Kodira C, Tu Z, Loftus B, Xi Z, Megy K, Grabherr M, Ren Q, Zdobnov EM, Lobo NF, Campbell KS, Brown SE, Bonaldo MF, Zhu J, Sinkins SP, Hogenkamp DG, Amedeo P, Arensburger P, Atkinson PW, Bidwell S, Biedler J, Birney E, Bruggner RV, Costas J, Coy MR, Crabtree J, Crawford M, deBruyn B, DeCaprio D, Eiglmeier K, Eisenstadt E, El-Dorry H, Gelbart WM, Gomes SL, Hammond M, Hannick LI, Hogan JR, Holmes MH, Jaffe D, Johnston JS, Kennedy RC, Koo H, Kravitz S, Kriventseva EV, Kulp D, LaButti K, Lee E, Li S, Lovin DD, Mao C, Mauceli E, Menck CFM, Miller JR, Montgomery P, Mori A, Nascimento AL, Naveira HF, Nusbaum C, O'Leary S, Orvis J, Pertea M, Quesneville H, Reidenbach KR, Rogers Y, Roth CW, Schneider JR, Schatz M, Shumway M, Stanke M, Stinson EO, Tubio JMC, VanZee JP, Verjovski-Almeida S, Werner D, White O, Wyder S, Zeng Q, Zhao Q, Zhao Y, Hill CA, Raikhel AS, Soares MB, Knudson DL, Lee NH, Galagan J, Salzberg SL, Paulsen IT, Dimopoulos G, Collins FH, Birren B, Fraser-Liggett CM, Severson DW. 2007. Genome Sequence of *Aedes aegypti*, a Major Arbovirus Vector. *Science* 316: 1718-1723, doi {10.1126/science.1138878} {http://www.sciencemag.org/cgi/content/abstract/316/5832/1718}

NYCDOHMH [New York City Department of Health and Mental Hygiene]. 2004. Comprehensive mosquito surveillance & control plan [Internet]. Available from New York City Department of Health and Mental Hygiene, New York [accessed March 20, 2009]. {http://nyc.gov/health}

Pates H, Curtis C. 2005. Mosquito behavior and vector control. *Annu. Rev. Entomol.* 50: 53-70.

Perera MD, Hemingway J, Karunaratne SHPP. 2008. Multiple insecticide resistance mechanisms involving metabolic changes and insensitive target sites selected in anopheline vectors of malaria in Sri Lanka. *Malaria J.*. 7: 168.

Pinto J, Lynd A, Vicente JL, Santolamazza F, Randle NP, Gentile G, Moreno M, Simard F, Charlwood JD, Rosário VE, Caccone A, Torre A, Donnelly MJ. 2007. Multiple origins of knockdown resistance mutations in the afrotropical mosquito vector *Anopheles gambiae*. PLoS ONE 2(11): e1243. doi: 10.1371/journal.pone.0001243.

Roozendal JA, Van Hoof JPM, Voorham J, Oostburg BFJ. 1989. Behavioral responses of *Anopheles darlingi* in Suriname to DDT residues on house walls. *J. Amer. Mosq. Control* Assoc. 5: 339-350.

Rutledge CR, Day JF, Lord CC, Stark LM, Tabachnick WJ. 2003. West Nile Virus infection rates in *Culex nigripalpus* (Diptera: Culicidae) do not reflect transmission rates in Florida. *J. Med. Entomol.* 40: 253-258.

Sardelis MR, Turell MJ, Dohm DJ, O'Guinn ML. 2001. Vector competence of selected North American *Culex* and *Coquillettidia* mosquitoes for West Nile Virus. *Emerg. Infect. Dis.* 7: 1018-1022.

Shang QL, Liang P, Gao XW. 2008. Cloning, developmental and tissue-specific expression of y-aminobutyric acid (GABA) receptor alpha2 subunit gene in *Spodoptera exigua* (Hübner). *Pest. Biochem. Physiol*. 93: 1-7.

Scholte EJ, Ng'habi K, Kihonda J, Takken W, Paaijmans K, Abdulla S, Killeen GF, Knols BGJ. 2005. An entomopathogenic fungus for control of adult African malaria mosquitoes. *Science* 308: 1641.

Smartt CT, Erickson JS. 2008. Bloodmeal-induced differential gene expression in the disease vector *Culex nigripalpus* (Diptera: Culicidae). *J Med Entomol*. 45(2): 326-330.

Vaughan A, Hawkes N, Hemingway J. 1997. Co-amplification explains linkage disequilibrium of two mosquito esterase genes in insecticide-resistant *Culex quinquefasciatus*. *Biochem J.* 325: 359-65.

Vaughan A, Hemingway J. 1995. Cloning and sequence of the full-length cDNA for a major insecticide resistance gene worldwide in the mosquito *Culex quinquefasciatus*. *The Amer. Soc. Biochem. Molec. Biol.* 270: 17044-17049.

Weill M, Lutfalla G, Mogensen K, Chandre F, Berthomieu A, Berticat C, Pasteur N, Philips A, Forte P, Raymond M. 2003. Insecticide resistance in mosquito vectors. *Nature* 423: 136-137.

WHO [World Health Organization]. 1979. Resistance of vectors and reservoirs of disease to pesticides: Twenty-second report of the WHO expert committee on insecticides. World Health Org. Tech. Report Series. No. 585.

BIOGRAPHICAL SKETCH

Shainnel Onaleigh Eans was born in Mount Vernon, New York. Her greatest inspiration throughout her life is her mother, who is a single mother that has always worked hard to provide for the family. Her mother always encouraged her children to follow their dreams wherever they may lead. This would play a large role in Shainnel's life and future ambitions. Since a young age, Shainnel has been interested in two main topics: the sciences and languages. One of her goals in life is to become proficient in at least five languages, excluding English. Currently, she knows Spanish and Japanese and is now planning what her next languages will be.

Shainnel graduated from the University of Florida in 2007 with a Bachelor of Science in zoology and a minor in Japanese. After her studies in zoology, she determined that she was most interested in invertebrates and parasitology. Shainnel chose to continue her education at the University of Florida for a Master of Science in the Department of Entomology and Nematology where she studies insecticide resistance genes in the mosquito species *Culex nigripalpus*. She hopes to one day apply her strong background in the sciences and knowledge of languages in future ambitions.