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Association of Esterases in Resistance to Naled and Resmethrin in the Southern House Mosquito, Culex quinquefasciatus

Jennifer R. Gordon
Louisiana State University and Agricultural and Mechanical College

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ASSOCIATION OF ESTERASES IN RESISTANCE TO NALED AND RESMETHRIN IN THE SOUTHERN HOUSE MOSQUITO, *CULEX QUINQUEFASCIATUS*

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science

in

The Department of Entomology

by

Jennifer R. Gordon
B.S. (Entomology), Purdue University, 2008
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DEDICATION

I would like to dedicate this thesis to the love and support I received from my mother Michelle, and my father, David. The pureness of their love has carried me throughout my life. Without knowing such unconditional love, I would have ended up like so many of my peers back home and this thesis would never have been.
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ABSTRACT

The vector-competency and its affinity for humans make *C. quinquefasciatus* an important target of mosquito abatement programs. Whenever such control programs are implemented, protocols to monitor frequencies and mechanisms of resistance to the insecticides used are necessary to optimize the efficacy of the management strategy and to slow development of resistance to the insecticides used. In the current study, susceptibilities to the two adulticides used by EBRMARC (naled and resmethrin) were monitored using field-collected mosquitoes and a topical and contact bioassay, respectively. My hypothesis was that esterase-mediated enhanced metabolism conferred resistance to both insecticides in populations of *C. quinquefasciatus* from EBR Parish. To test this hypothesis, esterase activities from field-collected mosquitoes were monitored using a model substrate, and esterases were visualized using native polyacrylamide gel electrophoresis. In addition, naled was tested as a synergist of the toxicity of resmethrin to further explore the relationship between esterases and increased frequencies of resistance, and to examine the use of naled as a possible countermeasure to resmethrin resistance. The results from this study will allow management strategies for populations of *C. quinquefasciatus* to be optimized, and provide a foundation for further studies exploring esterase inhibitors as synergists of pyrethroid toxicity.
CHAPTER 1. INTRODUCTION

Insecticides are important tools for management of insect vectors of human disease, which cause tremendous human morbidity and mortality, and affect hundreds of millions of people throughout the world. Mosquitoes in the genus *Anopheles* infect 300-500 million people every year with malaria-causing *Plasmodium* species. Tragically, 66 million of these cases occur in children between the ages of 0-4 y and 430,000 to 680,000 die (Snow *et al.*, 1999). When the World Health Organization (WHO) first used dichlorodiphenyltrichloroethane (DDT) in a global program to eliminate malaria by controlling the vector *Anopheles*, the insecticide was heralded as a savior of its time (McGinn, 2002). No other single control strategy or integrated management program had ever worked so effectively at controlling mosquitoes. Through 1966, over 500 million people were saved through suppression of vector populations with DDT (Shiff, 2002). Similarly, use of insecticides in agriculture has led to dramatic increases in food and fiber production. Once the insecticidal properties of Paris Green and London Purple were first discovered in the late 19th century, insecticides became an integral component in efforts to feed the human race (Perkins & Holochuck, 1993). In the United States alone, 500,000 tons of pesticides, at an annual cost of $4.1 billion, are sprayed to control insects, weeds and plant pathogens. Estimates suggest that application of these pesticides reduced crop loss by 10 %, and for every $1 spent on insecticides, $4 is returned (Pimentel *et al.*, 1993).

No matter how positive the effects may be from spraying an insecticide, there are certain risks associated with a toxicant being sprayed into the environment, especially the potential for widespread environmental contamination and non-target exposure. Residues were everywhere from the unadulterated use of DDT, and the public's fears about the possible deleterious effects of DDT contributed to a ban on the use of the insecticide in 1972. However, people began to take notice of the toxic effect insecticides could have on non-target organisms long before the
insecticidal properties of DDT were discovered. In 1881, massive killings of honey bees on pear trees were reported after exposure to arsenic (Caron, 1999). Not only are beneficial insect populations affected, but also insecticide residues are found on our foods. For example, a study sampling 100 different pesticides in over 100 different fruit drinks from 15 countries throughout the world found that the majority (> 80 %) was contaminated. Whereas the majority of pesticides found were fungicides, 22 % of samples tested were positive for malathion, a common organophosphate used to control various insect pests (Garcia-Reyes et al., 2008). In many respects, concerns regarding insecticide exposure and contamination provided the foundation for the environmental movement in the late 1960's. In 1962, Rachel Carson wrote Silent Spring in which she gave a testament to the outcome of widespread and reckless use of DDT (Carson, 1962). The emotional outcry that followed Carson's book contributed to the cessation of the production and use of DDT to control insect pests in the U.S. However, by the time DDT was banned, another problem intrinsic to insecticide use, insecticide resistance, had developed in populations of mosquitoes that had greatly compromised the continued efficacy of the insecticide.

Insecticide resistance is an excellent example of evolution in action: anything that kills, selects for resistance. Resistance is defined as the “ability in a strain of insects to tolerate doses of toxicants which would prove lethal to the majority of individuals in a normal population of the same species" (Anonymous, 1957). The first published example of resistance was observed in San Jose scale to sulphur-lime (Melander, 1914; 1915). Much later, resistance to DDT in mosquitoes was found in Florida salt marsh mosquitoes, Aedes taeniorhynchus and sollicitans, as a result of intensive spraying from 1945 to 1950 (Deonier & Gilbert, 1950). At the time when Melander first wrote about resistance, entomologists misunderstood the underlying mechanisms involved, which lead Melander to state, "We do not know that such an acquired immunity affects
subsequent generations, although it would not be inconceivable that arsenic antibodies are passed on by the mother into the egg to give the offspring some initial immunity." Today, three mechanism of resistance are known to be widely expressed, existing as heritable traits passed in Mendelian fashion from the parents to the offspring (Oppenoorth & Welling, 1976).

For over 40 years, mosquito abatement programs have applied extreme selection pressure on populations of mosquitoes through application of organophosphate and pyrethroid insecticides, and use of these two classes of insecticides has led to increased frequencies of resistance (Brook et al., 2001). The lack of resistance monitoring protocols has reduced efficacies of both classes of insecticides for mosquito control (Chandre et al., 1999). Despite reductions in efficacious insecticides, controlling the vector is the best way of reducing incidences of disease. Only through knowing the mechanism(s) of resistance can mosquito abatement programs slow development of resistant populations and develop countermeasures in populations where resistance is already established.

In the present study, susceptibilities to the two adulticides used by EBRMARC (naled and resmethrin) were monitored using field-collected mosquitoes and a topical and contact bioassay, respectively. My hypothesis was that esterase-mediated enhanced metabolism conferred resistance to both insecticides in populations of C. quinquefasciatus from EBR Parish. To test this hypothesis, esterase activities from field-collected mosquitoes were monitored using a model substrate, and esterases were visualized using native polyacrylamide gel electrophoresis. In addition, naled was tested as a synergist of the toxicity of resmethrin to further explore the relationship between esterases and increased frequencies of resistance, and to examine the use of naled as a possible countermeasure to resmethrin resistance. The results from this study will allow management strategies for populations of C. quinquefasciatus to be optimized, and provide a foundation for further studies exploring esterase inhibitors as synergists of pyrethroid toxicity.
1.1 References


CHAPTER 2. REVIEW OF LITERATURE

2.1 Introduction

Mosquito-vectored diseases cause tremendous human morbidity and mortality affecting hundreds of millions of people throughout the world. Mosquitoes in the genus *Anopheles* infect 300-500 million people every year with malaria-causing *Plasmodium* species, and one million of those people die (McGinn, 2002). Such high rates of infection and mortality make malaria one of the most important human diseases in the world. In addition, dengue virus, the most commonly vectored arbovirus, is a deadly flavivirus associated with mosquitoes in the genus *Aedes*, and affects 50-100 million people annually (Guha-Sapir, & Schimmer, 2005). Whereas dengue virus was previously classified as a tropical disease, the virus is now appearing in subtropical regions and could have important public health concerns for citizens of Louisiana. As of August 7, 2010, 25 cases from autochthonous transmission of dengue virus have occurred in Florida, with an additional 57 cases reported by people returning from travel in a dengue endemic country (Kramer, 2010). Finally, there are a host of local examples of mosquito-vectored arboviruses that are familiar to residents of the southern U.S. For example, West Nile virus in Louisiana, which is primarily vectored by *Culex quinquefasciatus* in humans, threatened millions of residents where 990 human cases have been reported since 2001, including 62 deaths (Center for Disease Control and Prevention, 2009). With the plethora of diseases potentially vectored by many different species of mosquitoes, an understanding of the biology of these vectors is vital for abatement programs.

2.2 Biology, Ecology and Taxonomy of *C. quinquefasciatus*

Populations of *C. quinquefasciatus* mosquitoes have multiple, overlapping generations and grow to immense numbers in a short amount of time (Darsie & Ward, 2005). Female *C. quinquefasciatus* are typically monogamous, mating only once (Craig 1967), and require a blood
meal for vitellogenesis. They feed on a multitude of hosts including avians and mammals, and the gonotrophic cycle typically lasts two to seven d after the blood meal (Eldridge, 2005). They then lay egg rafts containing 100 or more eggs on the surface of water that is highly organic such as septic ditches, sewer drains and livestock run off ponds (Laird 1988). The life cycle of these mosquitoes is temperature dependent; however, neonates generally emerge within 24 to 36 h after oviposition, and larvae will feed for seven to 14 d and pass through four instars. The end of the fourth stadium is followed by pupation, which can last two to three d and ends with eclosion of an adult mosquito (Darsie & Ward 2005). Males will emerge first to allow their genitalia to rotate a full 180° (Clements, 1992), and females will be receptive to mating within two days of emergence (Eldridge, 2005). Both males and females require nectar for metabolic energy. Given that C. quinquefasciatus rapidly produce dense populations and have a wide host range, they are potential nuisances and, as described above, important disease vectors.

Mosquitoes of the genus Culex can be difficult to differentiate to species. The Southern house mosquito, C. quinquefasciatus, is closely related to C. pipiens, the Northern house mosquito. Differentiating between the two mosquitoes has been a challenge to culicid taxonomists (Mattingly, 1967) and debate continues over the status of C. quinquefasciatus as a separate species from C. pipiens. Crabtree et al. (1997) developed a technique using the polymerase chain reaction that clearly distinguishes the two species using a 600 bp sequence unique to C. pipiens. Besides genetic difference, the two species exist in two distinct climatic regions of the United States, as their common names imply, with a middle region where the two species overlap and occasionally hybridize (Darsie & Ward, 2005).

2.3 Mosquito Abatement and Insecticide Resistance

Traditionally, most disease control programs have used insecticides to prevent infection by killing the mosquito before transmission of pathogen can occur. For example, in 1955, the
World Health Organization (WHO) launched the “Global Malaria Eradication Program” and created a plan to use DDT to stop *Anopheles* mosquitoes from spreading the pathogens that cause malaria (McGinn, 2002). The plan worked well. In the next 10 years, DDT saved an estimated 525 million people from malaria. However, by 1966, 15 species of anopheline mosquitoes were resistant to DDT (Shiff, 2002).

Mosquito control is also a local enterprise. A chemical strategy to manage mosquitoes was launched in East Baton Rouge (EBR) Parish when residents approved a tax on January 20, 1979 to form the EBR Mosquito Abatement and Rodent Control (EBRMARC; EBR-GW, 2008). Shortly thereafter, mosquito abatement became a government-controlled operation targeting culicid larvae and adults. Today, *C. quinquefasciatus* is the main vector targeted by EBRMARC’s larvicide program. Typically, technicians apply larvicide either by hand or from trucks and target various aqueous sites. The larvicides they use include: *Bacillus sphaericus* (VectoLex® WDG), *Bacillus thuringiensis israelensis* (VectoBae® 12AS; G), methoprene (Altosid® pellets), and biodegradable oils (Agniquest®, GB 1111; EBR-GW, 2008). In addition to immatures, adult populations are also targeted for control.

Pyrethroids and OPs are the two classes of adulticides approved for mosquito control by the Environmental Protection Agency (Rose, 2001). The OPs are phosphate esters that inhibit acetylcholinesterase (AChE), thus preventing degradation of the neurotransmitter acetylcholine. The insect then becomes paralyzed and dies as concentrations of acetylcholine build in the synapse (O’Brien 1967). Pyrethroids are carboxyl esters and kill insects by altering the inactivation of voltage-gated sodium channels along the axon of the neuron (Soderlund & Bloomquist, 1989). The two current adulticides applied by EBRMARC are naled (1,2-dibromo-2, 2-dichloroethyl dimethyl phosphate), an OP, and resmethrin ((5-benzyl-3-furyl) methyl 2,2-dimethyl-3- (2-methylpropenyl) cyclopropanecarboxylate), a pyrethroid (EBR-GW, 2008).
Regardless of the mode of action, anything that kills selects for resistance, and, as mentioned before, resistance is a recurrent problem in mosquito abatement programs.

Resistance is defined as the “ability in a strain of insects to tolerate doses of toxicants which would prove lethal to the majority of individuals in a normal population of the same species” (Anonymous, 1957). A population of mosquitoes becomes resistant as an insecticide kills a majority of susceptible members, leaving increased frequencies of mosquitoes with heritable mutations conferring survival. Whereas the first observed cases of this type of selection were in San Jose scale to sulphur-lime (Melander, 1914; 1915), the first recorded incident of culicid resistance to insecticides was found in Florida salt march mosquitoes, Aedes taeniorhynchus and sollicitans, to DDT after intensive spraying from 1945 to 1950 (Deonier & Gilbert, 1950). Resistance to insecticides in mosquitoes has expanded to include over 50 anopheline and over 30 culicine mosquito species, and encompasses all classes of insecticides. Populations of C. quinquefasciatus are particularly resistant to a wide assortment of chemical classes, and occur over many geographic regions (WHO, 1992).

2.4 Mechanisms of Resistance

Advantageous genetic mutations confer resistance to insecticides by one of three major mechanisms: reduced cuticular penetration, reduced target-site sensitivity and enhanced metabolism of the insecticide. Reduced cuticular penetration results in a low level of resistance by slowing the rate at which an insecticide can traverse the insect cuticle. For example, cuticular penetration of DDT was reduced by 1.5-fold in a resistant (91-R) stain of Drosophila melanogaster compared to the susceptible, Canton-S strain (Strycharz 2010). In a similar study investigating penetration of the pyrethroid deltamethrin into the cotton bollworm, Helicoverpa armigera, 50 % of the applied insecticide was absorbed into larvae after 1 h in the susceptible strain, but penetration of 50 % required 6 h in the resistant strain (Ahmad et al., 2006).
Sometimes reduced penetration through the cuticle results only in a delayed knockdown of the insect (Oppenoorth & Welling, 1976). Wood et al. (2010), found a positive correlation between cuticle thickness and the time for knockdown in resistant *Anopheles funestus*.

The cellular mechanism underlying reduced penetration is unknown; however, in some cases altered activity and expression of membrane bound transporter proteins has been implicated. P-glycoproteins (pgp) are ATP driven transporter proteins associated with efflux of xenobiotics from within the cell. When expression of pgp was compared in various body regions (i.e., cuticle, fat body and midgut) between resistant and susceptible strains of the tobacco budworm, *Heliothis virescens*, levels of pgp were higher in the resistant strains and most concentrated in the cuticle (Lanning et al. 1996). Another possible mechanism of decreased cuticular penetration involves an increase in laccase genes. Laccases are enzymes whose function in insects is not well understood, but they are believed to be involved in cuticular sclerotization. The laccase 2 gene (*CpLac2*) has been sequenced, and the gene's expression measured in resistant and susceptible *C. pipiens pallens*. Penetration-resistant insects have over 20-fold greater expression of this gene compared to susceptible members of this species (Pan et al., 2009). Whereas reduced cuticular penetration results in significant loss of susceptibility, more frequently, maximal resistance is associated with (co)expression of reduced target site sensitivity or enhanced metabolism.

Point mutations associated with target site resistance to a number of insecticide classes are now known (ffrench-Constant et al., 1998); thus, understanding of the molecular aspects of reduced target site sensitivity has increased. For example, much is known about mutations that cause knockdown (kdr) and super-kdr resistance to pyrethroids and DDT (Knipple et al., 1994). The genetic change associated with kdr is a single point mutation in the gene encoding a leucine within the voltage gated sodium channel. The specific mutation at this site is dependent on the
insect species. For example, the mutation is Leu → Phe in the house fly, *Musca domestica* (Williamson *et al.*, 1996), Leu → His in *H. virescens* (Park & Taylor, 1997) and Leu → Ser in *C. pipiens* (Martinez-Torres *et al.*, 1999). The Leu → Phe mutation is found throughout the world and conveys a high level of resistance to both DDT and pyrethroids (Martinez-Torres *et al.*, 1999). In contrast, the Leu → Ser mutation conveys a high level of resistance to DDT but a low level to pyrethroids, and, until recently, had never been found in North America (Zhou *et al.*, 2009). The super-kdr resistance phenotype is associated with an additional point mutation (Met → Thr) that always occurs in conjunction with *kdr* (Williamson *et al.*, 1996). These mutations confer impressive (>11,000 fold) resistance to pyrethroids (Guerrero *et al.*, 1997). Only two point mutations (*kdr* and super-*kdr*) in sodium channels possibly occur because of the fitness cost associated with other structural alterations of this protein (ffrench-Constant *et al.*, 1998). On the other hand, at least nine independent point mutations are known to be associated with reduced sensitivity of acetylcholinesterase, the target site for OPs and carbamates (reviewed in Feyereisen, 1995). Point mutations in acetylcholinesterase confer high levels of resistance to these insecticides by interfering with the catalytic function of the enzyme. Reduced target site sensitivity has grave ramifications for pest management with insecticides, because once sensitivity of the target site has been reduced, all insecticides acting at that target site do so with reduced efficacy.

The third mechanism of resistance involves enhanced metabolism of the insecticide. Enhanced metabolism results from altered expression of one of three major classes of detoxifying enzymes: glutathione S-transferases (GST), P450 monooxygenases and esterases (EST). The GSTs catalyze attack of the tripeptide, glutathione, on electrophilic centers within lipophilic molecules (Chasseaud, 1979). Resistance conferred by GSTs results via conjugation, metabolism or sequestration of the insecticide (Ranson & Hemingway, 2005). The OPs were the
first class of insecticides that were shown to be directly detoxified by GSTs. Resistance to
tetrachlorvinophos in house flies was shown to result from conjugation of glutathione to the OP,
allowing for more rapid excretion and detoxication of the parent insecticide (Oppenoorth et al.,
1979). In rare cases, GSTs catalyze insecticide detoxication via mechanisms other than
conjugation with reduced glutathione. Thus, GSTs confer resistance to DDT by dechlorinating
the compound to DDE, thus reducing the insecticidal efficacy (Clark and Shamaan, 1984). In
addition, a pyrethroid was shown to be sequestered by GSTs in resistant populations of *Tenebrio
molitor* as evidenced by decreased GST activity to the substrate 1-chloro-2, 4-dinitrobenzene
(CDNB) while still having elevated glutathione concentrations after exposure to the insecticide
(Kostaropoulos *et al.* 2001).

A second family of insecticide detoxifying enzymes, the P450 monooxygenases, have
been well studied and are implicated as one of the main enzymes involved in the detoxication of
pyrethroids (Soderlund & Casida, 1977). Cytochrome P450 monooxygenases are membrane
bound and catalyze oxidation of a variety of endogenous substrates and xenobiotics (reviewed in
Feyereisen 1999). The malaria vector, *An. funestus*, was thought to have been under control, but
a re-emergence and subsequent outbreak of malaria from 1996-2000 resulted from cytochrome
P450-mediated resistance to pyrethroid insecticides (Amenya *et al.*, 2008). In resistant
*Drosophila*, an over-expressed gene, *Cyp6g1*, coding for a P450 enzyme confers resistance to
DDT as well as three neonicotinoids: imidaclorpid, acetamiprid and nitenpyram (Le Goff *et al.*, 2003).

Thirdly, esterases are a large, multi-gene family of enzymes important for the hydrolysis
of ester moieties in insecticides such as OP and pyrethroids (Dauterman, 1976). In resistant
*Pediculus capitis*, esterase-mediated enhanced metabolism conferred over 3.5- fold resistance to
an OP and a pyrethroid when compared to a susceptible strain (Gao *et al.*, 2006). Another study
investigating resistant peach aphid, *Myzus persicae*, showed that over expression of esterases resulted in 20-fold decrease of susceptibility to dimethoate (Needham & Devonshire, 1975).

No matter what enzyme is involved, enhanced metabolism of an insecticide involves either a qualitative or a quantitative change in expression. Thus, expression of a mutant, detoxifying enzyme with a heightened affinity for an insecticide can confer a high level of resistance. For example, in *C. tarsalis*, a mutant carboxylesterase is expressed with the increased ability to hydrolyze malathion (Ziegler *et al.* 1987). Similarly, a mutant GST with a unique activity (DDT dechlorinase) was first described by Clark and Shamaan (1984) in a DDT resistant strain of the house fly, *Musca domestica*. Finally, in resistant sheep blow fly, *Lucilia cuprina*, five point mutations are associated with an altered carboxylesterase with a heightened activity toward OPs (Newcomb *et al.*, 1997).

Overproduction of a wild-type insecticide-detoxifying enzyme by either gene amplification or upregulation is a more common form of enzyme-mediated resistance and occurs for all three classes of detoxifying enzymes (Hemmingway *et al.*, 1998). Gene amplification was first demonstrated for esterases in aphids (Devonshire & Moores, 1982), but has now been documented in Diptera, including mosquitoes, and other Hemipterans (Devonshire & Field, 1991). In effect, the enzymes act as "sequesterases" that have slow catalytic rates but act as a sink and prevent the insecticide from interacting with the intended target site. Mouches *et al.* (1986) originally estimated that there were 250 copies of an esterase gene in OP-resistant *C. quinquefasciatus*. In addition, Devonshire and Moores (1982) showed that 3% of the total proteins of *M. persicae* were esterases that conveyed cross-resistance to OP, carbamates and pyrethroids. More commonly, upregulation of a gene for a metabolically active enzyme occurs (Hemingway & Ranson, 2000). Upregulation, which occurs with all three enzyme families, happens when an alteration in the transcription of the gene causes an over-expression of a wild-
type protein (Hemingway et al. 1998).

In addition to these three mechanisms, over-expression of the transporter protein, p-glycoprotein, is considered by some as a fourth mechanism of resistance; however, at this point, evidence is circumstantial. By inhibiting pgp in *H. virescens*, the lethal dose that kills 50% of the population (i.e., the LD$_{50}$) was decreased by 12.5-fold (Lanning et al., 1996). In a similar study using larval *C. pipiens*, an increase in susceptibilities to ivermectin, endosulfan and cypermethrin, but not chlorpyrifos, was recorded after exposure to the pgp inhibitor, verapamil (Buss et al., 2002). More recently, pgp has been implicated in resistance to DDT in *D. melanogaster* as well. Susceptibility to DDT in the resistant (91-R) strain of fruit flies was increased by 10-fold after exposure to verapamil (Strycharz, 2010). Whereas increased toxicity in the presence of a pgp inhibitor is suggestive of p-glycoprotein involvement in resistance, more work is needed to evaluate the specifics of this potential mechanism.

### 2.5 Resistance Management

Application of any insecticide selects for insecticide resistance. Thus, resistance management strategies are necessary components of control. Two major components of resistance management are to survey insecticide susceptibility in the target pest, and, where resistance occurs, explore mechanisms that underlie resistance. Once the mechanism of resistance has been established, countermeasures can be developed. For instance, to slow development of resistance due to expression of reduced target site sensitivity in a population, rotation of different classes of insecticides (acting at different target sites) must be used. Implementation of an insecticide rotation program alternating endosulfan, an OP and a pyrethroid in Canadian fruit crops against *Grapholita molesta* decreased frequencies of resistance to an OP from 55 to 14%, and to pyrethroids from 30 to 10% (Kanga et al., 2003). Similarly, application of acephate onto cotton was shown to reduce first through third instars of
tarnished plant bugs, *Lygus hesperus* (Fontenot, 2009). In contrast, if enhanced metabolism is the mechanism of resistance, rotation of chemistries might not necessarily be effective. However, synergism using inhibitors of said enzymes may be one countermeasure.

Insecticide synergists are compounds that greatly enhance toxicity of an insecticide when used in combination with an insecticide. Synergism of insecticide toxicity occurs when a compound that inhibits insecticide metabolism is used in conjunction with the insecticide. For example, piperonyl butoxide (PBO) synergizes the toxicity of a number of insecticides believed to be detoxified by P450s (reviewed in Casida, 1970). In the German cockroach, *Blattella germanica*, mortality was increased in the presence of PBO (Scott *et al.*, 1990). Inhibition of insecticide metabolism also may result in antagonism of toxicity. Thus, whereas PBO synergized the toxicity of a pyrethroid in two malaria vectors, the toxicity of an OP was antagonized in the same mosquitoes by inhibiting the activation of the OP to the more lethal oxon (Perera *et al.* 2008). Monooxygenases are not the only class of detoxifying enzyme that can be synergized with an enzyme inhibitor. Strains of *C. pipiens* from China were more susceptible to OPs when treated with an esterase inhibitor, S, S, S-tributyl phosphorotrithioate (DEF; Qiao *et al.*, 1998). Yet another study showed that DEF synergized the toxicity of cypermethrin, a pyrethroid insecticide, in *M. domestica* (Zhang *et al.*, 2007).

### 2.6 Justification of Study

A previous study showed that multiple mechanisms are expressed in populations of *C. quinquefasciatus* from southern Louisiana that are resistant to OPs and pyrethroids (Stancil, 2000); however, the same study found an association between esterases and resistance. In EBR Parish, decreased susceptibilities to malathion (from 9.2 to 78.1-fold) and resmethrin (from 1.6 to 3.2-fold) were reported in populations of *C. quinquefasciatus* and resistance was associated with increased esterase activity (Stancil 2000). Similarly, in populations of mosquitoes from
Florida and Alabama, esterases, along with other enzymes and target site mutations, were found to confer resistance to OPs and pyrethroids (Liu et al., 2004; Xu et al., 2005).

The vector-competency and its affinity for humans make *C. quinquefasciatus* an important target of mosquito abatement programs. Whenever such control programs are implemented, protocols to monitor frequencies and mechanisms of resistance to the insecticides used are necessary to optimize the efficacy of the management strategy and to slow development of resistance to the insecticides used. In the current study, susceptibilities to the two adulticides used by EBRMARC (naled and resmethrin) were monitored using field-collected mosquitoes and a topical and contact bioassay, respectively. My hypothesis was that esterase-mediated enhanced metabolism conferred resistance to both insecticides in populations of *C. quinquefasciatus* from EBR Parish. To test this hypothesis, esterase activities from field-collected mosquitoes were monitored using a model substrate, and esterases were visualized using native polyacrylamide gel electrophoresis. In addition, naled was tested as a synergist of the toxicity of resmethrin to further explore the relationship between esterases and increased frequencies of resistance, and to examine the use of naled as a possible countermeasure to resmethrin resistance. The results from this study will allow management strategies for populations of *C. quinquefasciatus* to be optimized, and provide a foundation for further studies exploring esterase inhibitors as synergists of pyrethroid toxicity.

2.7 References


Stycharz, J.P. (2010). Polygenic resistance in the highly DDT-resistant 91-R strain of *Drosophila*
melanogaster involves decreased penetration, increased metabolism and direct excretion of DDT (Masters thesis, University of Massachusetts- Amherst, 2010).


CHAPTER 3. ASSOCIATION OF ESTERASES IN RESISTANCE TO NALED AND RESMETHRIN IN THE SOUTHERN HOUSE MOSQUITO, CULEX QUINQUEFASCIATUS

3.1 Introduction

Mosquito-vectored diseases cause morbidity and mortality in hundreds of millions of people throughout the world. Mosquitoes in the genus Anopheles infect 300-500 million people every year with malaria-causing Plasmodium species, and one million of those people die (Snow et al., 1999). Such high rates of infection and mortality make malaria one of the most important human diseases in the world. In addition, dengue virus, the most commonly vectored arbovirus, is a deadly flavivirus associated with mosquitoes in the genus Aedes and affects 50-100 million people annually (Guha-Sapir, & Schimmer, 2005). Whereas dengue virus was primarily classified as a tropical disease, the virus now has important public health concerns for citizens of the Southern U.S. As of August 7, 2010, 25 cases from autochthonous transmission of dengue virus have occurred in Florida, with an additional 57 cases reported by people returning from travel in a dengue endemic country (Kramer, 2010). In addition to dengue, there are more familiar and pressing local examples of mosquito-vectored arboviruses as well. West Nile virus, primarily vectored to humans by the Southern house mosquito, Culex quinquefasciatus, threatens millions of residents of Louisiana where 990 human cases have been reported since 2001, including 62 deaths (Center for Disease Control and Prevention [CDC], 2009). With the plethora of diseases potentially vectored by many different species of mosquitoes, abatement programs focused on control of culicids are vital.

Most disease control programs use insecticides to prevent infection by killing the mosquito before transmission of pathogens can occur. For example, in 1955, the World Health Organization (WHO) launched the “Global Malaria Eradication Program” and created a plan to use DDT to stop Anopheles mosquitoes from spreading the pathogens that cause malaria (Snow
et al., 1999). The plan worked well. By 1966, DDT had saved an estimated 525 million people from malaria; however, during that same period, 15 species of anopheline mosquitoes became resistant to DDT (Shiff, 2002). On a local level, residents of East Baton Rouge (EBR) Parish approved a tax on January 20, 1979 to form the East Baton Rouge Mosquito Abatement and Rodent Control (EBRMARC; EBR-GW, 2008). Shortly thereafter, mosquito abatement became a government-controlled operation targeting culicid larvae and adults.

The main vector targeted by EBRMARC's larval abatement program is C. quinquefasciatus. Typically, technicians apply larvicide either by hand or from trucks and target various aqueous habitats along roadsides. Larvicides used include: Bacillus sphaericus (VectoLex® WDG), Bacillus thuringiensis israelensis (VectoBac® 12AS; G), methoprene (Altosid® pellets), and biodegradable oils (Agnique®, GB® 1111; EBR-GW, 2008). In addition to immatures, populations of adult mosquitoes are targeted for control.

Pyrethroids and OPs are the two classes of adulticides approved for mosquito control by the Environmental Protection Agency (Rose, 2001). The OPs are phosphate esters that inhibit acetylcholinesterase (AChE), thus preventing the enzyme from degrading the neurotransmitter acetylcholine. The insect then becomes paralyzed and dies as concentrations of acetylcholine build in the synapse (O’Brien 1967). Pyrethroids are carboxyl esters that kill insects by altering the inactivation of voltage-gated sodium channels along the axon of the neuron (Soderlund & Bloomquist, 1989). The two adulticides applied by EBRMARC are naled (1,2-dibromo-2,2-dichloroethyl dimethyl phosphate), an OP, and resmethrin ((5-benzyl-3-furyl) methyl 2,2-dimethyl-3-(2-methylpropenyl) cyclopropanecarboxylate), a pyrethroid (EBR-GW, 2008). The hypothesis tested here is that applications of their two, ester-containing insecticides by EBRMARC is selecting for esterase-mediated enhanced metabolism in populations of C. quinquefasciatus.
Esterases are enzymes associated with resistance to insecticidal esters, especially OPs and pyrethroids (Hemingway & Ranson, 2000). Enhanced metabolism by esterases occurs as a result of either a qualitative change or an overproduction of an enzyme. A qualitative change in an esterase occurs when a mutation of the gene results in an enzyme with heightened affinity for insecticide substrates. For example, a strain of *C. tarsalis* expresses a mutant carboxylesterase that has enhanced metabolism of malathion compared to a susceptible strain (Ziegler et al, 1987). Such qualitative changes may occur in conjunction with mutations that increase quantities of wild-type enzymes. Resistant head lice, *Pediculus capitis*, have a 13.3-fold increase in malathion carboxylesterase activity and a 3.9-fold increase in esterase activity against α-NA when compared to a susceptible strain (Gao et al., 2006). Overproduction of an esterase may occur by either gene amplification or upregulation. Such gene amplification in resistance has only been demonstrated for esterases. Devonshire and Moores (1982) showed that in resistant peach aphids, esterases account for 3 % of their total proteins. Similarly, in some resistant *C. quinquefasciatus*, esterases accounted for 0.4 % of the mosquito’s total protein (Karunaratne et al., 1993). The second way overproduction of wild-type enzymes occurs is through upregulation of esterase genes (Hemingway & Karunaratne, 1998).

The objectives of this study were to survey susceptibility in population of *C. quinquefasciatus* and investigate the association of esterases with naled and resmethrin resistance. Results suggest that susceptibilities to both insecticides varied across EBR Parish, and there is an association between esterase activities and frequencies of resistance in populations of this mosquito.

### 3.2 Materials and Methods

#### 3.2.1 Chemicals

Sodium phosphate (monobasic and dibasic; 99+ %), bis-acrylamide (ultra pure grade),
acrylamide (ultra pure grade), N, N, N', N'-tetramethylethylenediamine (TEMED; ultra pure grade), tris (biotechnology grade), ammonium persulfate (ACS grade), bovine serum albumin (BSA; biotechnology grade) and Coomassie Brilliant Blue G-250 (ultra pure) were purchased from AMRESCO (Solon, OH). Technical grade naled (1,2-dibromo-2,2-dichloroethyl dimethyl phosphate; 90 %) was donated by AMVAC (City of Commerce, CA), and resmethrin [(5-benzyl-3-furyl) methyl 2,2-dimethyl-3-(2-methylpropenyl) cyclopropanecarboxylate; 99.5 %] was purchased from Chem Service (West Chester, PA). Naphthyl acetates (α- and β-; 99+ %), Fast Blue B (90 %) and Fast Blue RR (90 %) salts were obtained from Sigma Aldrich (St. Louis, MO). Acetone (pesticide grade; 99.7 %), phosphoric acid (85 %), acetic acid (99.7 %), and glycine (reagent grade) were purchased from Fischer Scientific (Kansas City, MO). Bromophenol blue dye (ACS grade) was purchased from Eastman Kodak Company (Rochester, NY). Liver powder was ordered from M.P. Biomedical (Aurora, OH). Fish food (type “L”) was purchased from PETCO (TetraMin, Melle, Germany). Sodium citrate was purchased premixed from BD Vacutainer (Franklin Lakes, NJ).

3.2.2 Insects

A reference, susceptible strain of *C. quinquefasciatus* (SEBRING-S) was obtained from Harris County, Texas Mosquito Control and maintained in the Medical and Forensic Entomology Insectary in the Life Sciences Building of Louisiana State University. The United States Department of Agriculture- Agricultural Research Station in Gainesville, FL originally colonized the Sebring-S strain from mosquitoes collected in Sebring, FL (Johnsen, 2007). Adults were maintained in cages and were allowed to feed *ad libitum* from cotton balls dipped in a 10 % (w/v) sucrose solution. Female mosquitoes were fed through a parafilm membrane system that circulated hot water over chicken blood mixed with sodium citrate (0.109 M; Figure 1). After five days, a cup containing no less than 250 mL of “aged” tap water (i.e., exposed to the open air...
for 24 hr) was placed in the colony overnight to allow oviposition. The next morning, two to three egg rafts were transferred using screen mesh to 24 by 40 by 6 cm pans filled with aged tap water, and larvae were fed liver powder or baby fish food (2 mL; 1.25 %) daily. Pupae were segregated from larvae, and adults were allowed to emerge into mosquito breeding chambers (BioQuip Products, Co., Gardena, CA) that were held under a photoperiod of 10:14 (L:D), 21°C, and at 50% relative humidity.

Fig. 1. Apparatus for feeding adult, female mosquitoes. Chicken blood was warmed to 42°C by circulating hot water through a Rutledge Chamber with one opening covered with parafilm. Females were allowed to feed up to two hr.

For field-collections, more than 500 late instars and pupae were collected during 2009-2010 from septic ditches in East Baton Rough Parish, LA from areas treated with insecticides (Table 1). Insects were collected using a plastic scoop with an extended handle and transported
to the insectary in buckets containing water from the same site. Larvae and water were then transferred to pans in the insectary and immature insects were allowed to develop. Pupae were segregated, and adults were maintained as described above except that females were not fed.

Table 1. Application rate per year of two adulticides used for culicid control in East Baton Rouge Parish

<table>
<thead>
<tr>
<th>Site</th>
<th>Resmethrin</th>
<th>Naled</th>
<th>Collection Dates</th>
</tr>
</thead>
<tbody>
<tr>
<td>MARC</td>
<td>28</td>
<td>4.3</td>
<td>Nov 2009</td>
</tr>
<tr>
<td>HOOD1</td>
<td>32</td>
<td>16.5</td>
<td>Mar 2010</td>
</tr>
<tr>
<td>HOOD2</td>
<td>32</td>
<td>16.5</td>
<td>Apr 2010</td>
</tr>
<tr>
<td>THIB</td>
<td>20</td>
<td>2.8</td>
<td>June 2010</td>
</tr>
<tr>
<td>MINLOVE</td>
<td>22</td>
<td>8</td>
<td>Feb 2009; May 2010</td>
</tr>
</tbody>
</table>

Average frequencies based on records from EBRMARC for 2004 to 2009.

Field-collections were taken from various areas in East Baton Rouge Parish (Figure 2). The MARC site was located on EBRMARC's property and was the only site for which gravid water (i.e., a mixture of fish emulsion and water) was used to collect egg rafts. The two sites, HOOD1 and HOOD2, were approximately one block away from each other. However, HOOD1 was a long-lived septic leak in an abandoned lot of a residential area, whereas HOOD2 was a temporary septic leak located underneath a private home. The THIB site was located outside of Baton Rouge proper and was an open septic ditch. Finally, a recurrent bursting of septic pipes leading from a church created the site at MINLOVE.

3.2.3 Biological Assays

Susceptibilities to resmethrin and naled were measured using residue and topical assays, respectively. Unsexed, three to six day old adults weighing on average 2.6 mg were used in assays and were anesthetized with carbon dioxide prior to treatment. Mortality was scored after 18 h
and defined by the mosquito’s inability to right itself after 15 s. Prior to each assay, stock solutions (1 mM) of insecticide were made and serially diluted using acetone. One to five concentrations were tested per assay. A modified bottle bioassay (Plapp et al., 1987; Brogdon and McAllister, 1998) was chosen for the resmethrin exposure assays. Concentrations ranged from 0.25 to 1.10 µM for SEBRING-S or 0.75 to 9.00 µM for MINLOVE mosquitoes. Aliquots (1.0 ml) of resmethrin were added to 20 ml scintillation vials, which were rolled on the counter for 15 min then left to dry for two h. Next, 10 adult mosquitoes were placed, at random, into vials which were then sealed with cotton plugs. Vials coated with acetone served as a control. Each concentration was replicated nine to 15 times over three to five d for a total of 90-150 mosquitoes exposed per concentration. No control mortality was observed.

Fig. 2. Map of sites relative to one another in East Baton Rouge Parish (generated by Google Maps).

A topical bioassay was used to test susceptibility to naled. Five doses, ranging from 2.85
to 6.65 ng/insect for SEBRING-S, or eight doses, from 11.40 to 26.60 ng/insect for THIB, were applied using a microapplicator (Model M, ISCO©, Lincoln, NE) fitted with a Hamilton syringe. An aliquot (0.5 µL) of naled was applied to the thoracic dorsum of 10 mosquitoes. Each group of 10 was then sealed into a 20 mL scintillation vial with a cotton plug. Each dose was replicated three to nine times over one to three days for a total of 30 to 90 mosquitoes treated per dose. Mosquitoes treated with acetone served as a control. Again, no control mortality was observed.

For some sites, it was impractical to run a bioassay using a full range of concentrations; so, discriminating concentrations were calculated and used to measure frequencies of resistance. An LC$_{89}$ (1.97 nM) for resmethrin and an LD$_{98}$ (10.83 ng/insect) for naled were extrapolated using log-dose probit analysis from bioassay results from the SEBRING-S strain. Each concentration was administered as described above and depending on abundance of mosquitoes was replicated three to nine times per site over one to three days for a total of 30-90 mosquitoes treated. Mosquitoes treated with acetone served as a control. Abbott’s formula (Abbott, 1925) was used to correct for control mortality, which never exceeded 10%.

### 3.2.4 Synergism Bioassays

A bioassay was developed to investigate a possible synergistic effect of naled on the toxicity of resmethrin. Each determination used four 20 ml scintillation vials, and was replicated two to four times over one to four days. The first vial (resmethrin and naled) was coated with resmethrin and contained 10 adults that were pretreated with naled. The next three vials served as a series of controls. The first control (resmethrin only) was a vial coated with resmethrin that contained mosquitoes pretreated with acetone. The second control (naled only) was a vial coated with acetone and contained mosquitoes pretreated with naled. The third control (acetone only) was a vial treated with acetone that contained mosquitoes pretreated with acetone. All mosquitoes were allowed to dry for 30 min in sterile vials after topical treatment to minimize the
effect of acetone on cuticular penetration of resmethrin. Mortality was defined and scored as described above.

3.2.5 Biochemical Assays

Esterase activity toward α-NA was measured using the spectrophotometric assay of Gomori (1953) as modified by van Asperen (1962) and Grant et al. (1989). An individual, adult C. quinquefasciatus was homogenized in 100 µL of phosphate (phos) buffer (0.5 M, pH 7.0) using 10 strokes of a glass mortar and pestle, then centrifuged at 16,000 rpm for 10 min at 4 °C. Substrate solution was prepared by combining α-NA (2.26 mM final) with Fast Blue B (1.3 mM final) into phos buffer then filtered through Whatman® filter paper. Homogenate (20 µL) was combined with 200 µL of substrate solution and 30 µL of phos buffer then placed in a 96 well plate. Change in optical density was measured at 450 nm for 10 min using a microplate reader (SpectraMAX 190®, Molecular Devices, Sunnyvale, CA) and converted to units of µmoles min⁻¹ mg prot⁻¹ using the extinction coefficient 9.25 mM⁻¹ 250 µL⁻¹ (Grant et al., 1989). Protein concentration in the homogenate was determined using the method of Bradford (1979) using BSA as the standard.

Native polyacrylamide gel electrophoresis (PAGE) was used to visualize esterases of individual, adult mosquitoes using a vertical electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA) and 7.5 % polyacrylamide gels in Tris/glycine buffer (0.5 mM Tris, 77 mM glycine, pH 8.0; Gabriel 1971; Gabriel & Gersten 1992). An individual mosquito was homogenized in 25 µL of phosphate buffer (0.1 mM, pH 7.0), then combined with 5 µL of 6X tracking dye (0.25 % Bromophenol Blue and 40 % sucrose w/v) and centrifuged at 16000 g for 10 min at 4°C. Gels were loaded with 27 µL of supernatant and electrophoresed at a constant voltage (150 V) until the tracking dye was within 1 cm of the bottom of the gel. Gels were then submerged in 100 ml of 0.1 mM phosphate buffer (pH 7.0) containing 0.04 % (w/v) of α and β-
NA for 30 min in complete darkness. Next, the solution was decanted, combined with Fast Blue RR (0.1 % w/v) and then returned to the gel for an additional 20 min. Gels were then destained with double distilled water.

3.2.6 Data Analysis

Data from bioassays were analyzed using probit analysis and differences between slopes and x-intercepts were compared (Proc Ttest, SAS institute 2001). One-way analysis of variance (Proc GLM, SAS Institute 2001) was used to test for intersite differences between esterase activities and frequencies of resistance. Results from synergism bioassays were converted to synergism ratios (SR; mortality with naled and resmethrin/ mortality with resmethrin + mortality with naled alone) and t-tests were performed to determine if the ratios significantly differed from 1 (Proc Ttest, SAS Institute 2001).

3.3 Results

3.3.1 Susceptibility of C. quinquefasciatus to Naled and Resmethrin

Wild populations of mosquitoes collected in EBR Parish were resistant to resmethrin. The LC\textsubscript{50} of the field-collected MINLOVE population (1.9 µM) was 3.1 times greater than that of the reference-susceptible Sebring-S strain (0.66 µM; Figure 3). Based on the analysis of Sebring-S, a discriminating concentration of 1.97µM (ca. LC\textsubscript{89}) was chosen to estimate frequencies of resistance to resmethrin in field populations, which were moderate to high (> 39.2 to 94.2 %) in all field populations except for MARC (Table 2). The highest frequency of resistance was measured with HOOD1, in which 94.2 % of all adult mosquitoes survived exposure to the discriminating concentration. Collections from THIB had the next highest frequency of resistance (80.0 %), followed by HOOD2 (70.7 %) and MINLOVE (39.2 %). The collections from MARC had the lowest frequency of resistance (3.3 %) and were the only susceptible, wild population of mosquitoes tested.
Wild populations of mosquitoes were also resistant to naled. The LD$_{50}$ of the THIB population was 32.1 ng/insect and was 8.6 times greater than that of Sebring-S (3.74 ng/insect; Figure 4). Based on these data, a dose of 10.83 ng/insect (ca. LD$_{92}$) was chosen to estimate frequencies of resistance to naled in field collections. Unlike the frequencies of resistance to resmethrin, there was no inter-site variation to naled with all populations having high (i.e., $\geq 88.0\%$) frequencies of resistance. The population with the highest frequency of resistance to naled was THIB where 96.8 % of all adults survived exposure to the discriminating concentration. The field collections HOOD1 and MINLOVE had almost identical frequencies of resistance (93.9 and 93.3 % survival, respectively). Mosquitoes from HOOD2 had the lowest frequency of resistance (88.0 %) compared to the other field collections but still had a significantly increased frequency of resistance compared to Sebring-S.
Table 2. Frequencies of resistance (rFreq) to resmethrin and naled in field collections of *C. quinquefasciatus*

<table>
<thead>
<tr>
<th>Site</th>
<th>Resmethrin</th>
<th></th>
<th>Naled</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rFreq (± SE) df p-value</td>
<td></td>
<td>rFreq (± SE) df p-value</td>
<td></td>
</tr>
<tr>
<td>Sebring-S*</td>
<td>18.4 (± 11.3)a 7 -</td>
<td>7.60 (± 2.70)a 2 -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBRMARc</td>
<td>3.30 (± 3.40)a 2 0.9344</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HOOD2</td>
<td>70.7 (± 24.7)bc 2 0.0357</td>
<td>88.0 (± 4.70)b 5 &lt; 0.0001</td>
<td>93.9 (± 6.6)b 2 &lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>HOOD1</td>
<td>94.2 (± 5.80)b 2 &lt; 0.0001</td>
<td>96.8 (± 1.20)b* 1 &lt; 0.0001</td>
<td>93.9 (± 6.6)b 2 &lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>THIB</td>
<td>80.0 (± 5.80)b 2 0.0024</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MINLOVE</td>
<td>39.2(± 11.3)c 2 0.0468</td>
<td>93.3 (± 3.30)b 2 &lt; 0.0001</td>
<td>93.9 (± 6.6)b 2 &lt; 0.0001</td>
<td></td>
</tr>
</tbody>
</table>

Unless otherwise indicated, rFreq represents percent survival following exposure to a discriminating concentration of resmethrin (ca. LC$_{80}$; contact bioassay) or dose of naled (ca. LD$_{92}$; topical bioassay). Means within columns followed by the same letter are not significantly different from each other ($\alpha$ = 0.05; Tukey’s HSD test).

* Means were extrapolated from log-dose probit lines.

**Fig. 4.** Susceptibilities to naled of adult *C. quinquefasciatus* from Sebring-S (open circles) and THIB collections (closed squares). Arrow represents discriminating concentration used for estimating frequencies of resistance.
3.3.2 Synergism of the Toxicity of Resmethrin by Naled in *C. quinquefasciatus*

Naled had a synergistic effect on the toxicity of resmethrin in wild collections of mosquitoes; however, the effect was additive in tests with Sebring-S (Table 3). When applied separately to HOOD1 adults, naled killed 5.0 % of adults, and the dosage of resmethrin killed 15.3 %. However, when applied in tandem, 50.2 % of mosquitoes died, a SR of 2.97. Similarly, in mosquitoes from MINLOVE, naled alone killed 5.0 % and resmethrin alone killed 20.0 %, but the two insecticides together killed 60.0 % of all adults, a SR of 2.50. In contrast, in tests with adults from the Sebring-S strain, 15.6 % of adults died with naled alone and 21.1 % of adults with resmethrin, and the two insecticides combined killed 25.0 % of adult mosquitoes, a SR of 0.90.

Table 3. Synergism of resmethrin toxicity following pretreatment with naled

<table>
<thead>
<tr>
<th>Site</th>
<th>Treatment</th>
<th>Naled (± SE)</th>
<th>Resmethrin (± SE)</th>
<th>Nal and Res (± SE)</th>
<th>SRa (± SE)</th>
<th>df</th>
<th>p- value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sebring-S</td>
<td>15.6 ± 11.8</td>
<td>21.1 ± 9.1</td>
<td>25.0 ± 11.9</td>
<td>0.90 ± 0.57</td>
<td></td>
<td>3</td>
<td>0.2184</td>
</tr>
<tr>
<td>HOOD1</td>
<td>5.0 ± 5.0</td>
<td>15.3 ± 4.9</td>
<td>50.17 ± 10.6</td>
<td>2.97 ± 1.02</td>
<td>3</td>
<td>0.0500</td>
<td></td>
</tr>
<tr>
<td>MINLOVE</td>
<td>5.0 ± 5.0</td>
<td>20.0 ± 0.0</td>
<td>60.0 ± 0.0</td>
<td>2.50 ± 0.50</td>
<td>1</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>

Mean percent mortality (± SE) following treatment of adult mosquitoes with either naled or resmethrin alone or resmethrin following pretreatment with naled.

*S Synergism Ratio (SR) = percent mortality of adult mosquitoes pretreated with naled then exposed to resmethrin following pretreatment with naled / (percent mortality of adult mosquitoes after exposure to resmethrin + percent mortality of adult mosquitoes after exposure to naled).

3.3.3 Esterase Activities in *C. quinquefasciatus*

Esterase activities were significantly elevated in some (but not all) wild populations when compared to Sebring-S (Table 4). Adult mosquitoes from HOOD1 and MINLOVE had the highest esterase activity (3.39 µmol min⁻¹ mg prot⁻¹) toward α-NA. Similarly, elevated esterase activities were measured in collections of mosquitoes from THIB and MARC (2.80 and 2.03...
µmol min\(^{-1}\) mg prot\(^{-1}\), respectively) collections, and these activities were significantly higher than those measured in Sebring-S mosquitoes. However, esterase activities were relatively low in mosquitoes from HOOD2 (1.25 µmol min\(^{-1}\) mg prot\(^{-1}\)) and were not statistically different from the susceptible, Sebring-S strain. The frequency distributions of esterase activities were also similar between HOOD2 and Sebring-S, ranging between 0 to 3 µmol min\(^{-1}\) mg prot\(^{-1}\) (Figure 5). However, the distribution of activities in mosquitoes from MINLOVE was skewed to the right when compared to Sebring-S, and only 13.3 % of MINLOVE individuals overlapped with susceptible individuals.

**Table 4. Esterase activity of *C. quinquefasciatus* collected from East Baton Rouge Parish**

<table>
<thead>
<tr>
<th>Site</th>
<th>Esterase Activity (n = 30)</th>
<th>Mean (± SE)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sebring-S</td>
<td></td>
<td>1.08 (± 0.08)a</td>
<td>-</td>
</tr>
<tr>
<td>EBRMARC</td>
<td></td>
<td>2.03 (± 0.18)b</td>
<td>0.0002</td>
</tr>
<tr>
<td>HOOD2</td>
<td></td>
<td>1.25 (± 0.04)a</td>
<td>0.9918</td>
</tr>
<tr>
<td>HOOD1</td>
<td></td>
<td>3.39 (± 0.26)c</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>THIB</td>
<td></td>
<td>2.80 (± 0.14)b</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>MINLOVE</td>
<td></td>
<td>3.39 (± 0.32)c</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

Mean esterase activities (µmol min\(^{-1}\) mg prot\(^{-1}\) of α-naphthol produced) within columns not followed by the same letter are significantly different from each other (α = 0.05; Tukey’s HSD test).

**Fig. 5.** Distribution of esterase activity (µmol min\(^{-1}\) mg prot\(^{-1}\)) of adult *C. quinquefasciatus* individuals from Sebring-S (black; n = 30), HOOD2 (white; n = 30) and MINLOVE collections (grey; n = 30).
3.3.4 Electrophoretic Separation of Esterases in *C. quinquefasciatus*

Both inter- and intra-strain differences were observed in banding patterns of esterases and individual phenotypes were heterogeneous from both Sebring-S and MINLOVE collections (Figure 6). For Sebring-S (odd numbered lanes), the most prominent band was eb2 (57 KDa), which was evident in all individuals. A second band (eb3; 48 kDa) was visible in some but not all individuals (cf. lanes 1 and 7). Overall, MINLOVE mosquitoes (even numbered lanes) expressed more intense staining, especially for eb1 and eb3. In addition, expression of eb2, the most prominent band in Sebring-S, was variable in MINLOVE (cf. lanes 2 and 3). Finally, additional esterases were observed in all individuals from MINLOVE (eb1; 75 kDa) when compared to Sebring-S.

![Fig. 6](image_url)  
**Fig. 6.** Native PAGE of individual, adult mosquitoes homogenized in 30 µL of buffer/tracking dye solution from Sebring-S (odd lanes) and MINLOVE (even lanes) collections.

Esterases phenotypes among individuals from both the control and treated THIB groups were variable, with all mosquitoes expressing eb3 (Figure 7). All individual mosquitoes treated with acetone alone (lanes one through six) expressed a different phenotype with all bands visible in varying intensity, except eb2, which was lacking in one mosquito (cf. lane one). Individuals
exposed to an LD$_{50}$ of naled (lanes seven through 12) expressed a similar heterogeneity; however, eb1 was the occasional, lacking band (cf. lanes 10 and 11). In lanes 7-9, which represents individuals that appeared healthy after naled treatment, staining pattern was fairly consistent, especially for bands eb2 and eb3. For mosquitoes in lanes 10 and 11, which were scored as alive based on the criterion for mortality but were clearly intoxicated, staining intensity was reduced and eb1 was absent. Finally, the mosquito in lane 12 had uncoordinated movement and could not right itself; thus, was scored as dead. The phenotype of this mosquito lacked both eb1 and eb2, and eb3 was less intense than corresponding bands in lanes 7-9.

Fig. 7. Native PAGE of individual adult mosquitoes homogenized in 30 µL of buffer/tracking dye solution from THIB treated with acetone (control) or 38.1 ng/insect naled (LD$_{50}$; treated).

As with all gels, individual mosquitoes from MINLOVE expressed heterogeneous phenotypes in both the control and the treated groups (Figure 8). Individuals in the control group (lanes one through seven) all expressed bands eb1 and eb2, but intensity of expression was variable. As for eb3, the band seen in all three populations investigated thus far, only one individual from the control group exhibited expression (cf. lane one). All mosquitoes examined in the treated group (lanes eight through 14) were able to fly out of the vials without assistance.
after exposure to an LD$_{90}$ of naled for 18 h. Individuals from this group were heterogeneous as well, and overall expression of eb1 was similar to the control. Contrarily, mosquitoes from the treated group had highly variable expression of eb2 (cf. lanes eight, nine and 12), and all individuals examined expressed eb3 to some degree.

![Native PAGE](image)

**Fig. 8.** Native PAGE of individual adult mosquitoes homogenized in 30 µL of buffer/tracking dye solution from MINLOVE treated with acetone (control) or 95.2 ng/insect naled (LD$_{90}$; treated).

### 3.4 Discussion

Female *C. quinquefasciatus* are nuisance biters and known to vector arboviruses like West Nile virus (Godsey *et al.*, 2005). Thus, control programs targeting *C. quinquefasciatus* are necessary in regions with this culicid. Because of the frequency of insecticide application (Table 1), the hypothesis tested in this study is that resistance has developed in mosquitoes within EBR Parish. Further, because both insecticides used in this area are esters, an association between esterases and of resistance was examined.

Resistance to both resmethrin and naled exists in populations of *C. quinquefasciatus* from EBR Parish. In all populations examined, high frequencies of resistance (> 80 %) were detected to the OP, naled, whereas frequencies of resistance to resmethrin were more variable (3.3 to 94.2...
In a previous study, decreased susceptibilities to both an OP (9.2 to 78.1- fold) and a pyrethroid (1.6 to 3.2- fold) insecticide were detected in all populations of *C. quinquefasciatus* collected in the Parish (Stancil, 2000). The varied susceptibility to resmethrin might be due to the fact that the use of pyrethroids began in the late 1980's whereas OPs have been sprayed since the late 1960's (Stancil, 2000).

Resistance to resmethrin and naled are associated with increased esterase activity toward α-NA in some, but not all samples from wild collections. In previous studies, use of enzyme assays with non-insecticide (model) substrates as an indicator of enzyme involvement in metabolic resistance to insecticides has produced varied results. Studies have shown that increased activities of esterases toward α-NA is correlated with increased frequencies of resistance to insecticides. In the tobacco budworm, *H. virescens*, esterase activity was correlated ($r^2 = 0.87$) with increased frequencies of resistance in 15 populations examined across Louisiana (Zhou *et al.*, 1996; Harold & Ottea, 2000). However, in other studies, no such correlation was found (Brown & Brogdon, 1987; Ibrahim & Ottea, 1995). Thus, the toxicological significance of activities toward model substrates cannot be assumed. In the present study, collections from HOOD1, THIB and MINLOVE expressed high esterase activity and frequencies of resistance. However, MARC mosquitoes had heightened esterase activity but were susceptible to resmethrin. Finally, mosquitoes from HOOD2 had susceptible levels of esterase activity, but a high frequency of resistance. These findings may reflect heterogeneity in resistant mechanisms among populations and suggest that esterases are not solely involved in resistance to resmethrin in some populations. In contrast, a moderate correlation was measured between esterase activities and frequencies of resistance to naled ($r^2 = 0.47$). This finding suggests a possible relationship between esterase-mediated enhanced metabolism and resistance to naled in populations of *C. quinquefasciatus* but this association is not absolute. Also, these findings
suggest possible differences in the mechanisms of resistance for OPs and pyrethroids, and that cross-resistance is unlikely.

Qualitative differences in esterases among resistant and susceptible *C. quinquefasciatus* were observed and provide further evidence for the involvement of esterases in resistance. Banding patterns among individuals of all strains varied dramatically but were more intense in resistant mosquitoes, suggesting that esterases are associated with increased resistance but that multiple mechanisms have been selected in these populations. An esterase band (eb1) was observed in most resistant mosquitoes but was absent from a dying mosquito and individuals from the susceptible, Sebring-S strain. The appearance of this band is suggestive of some involvement in resistance, but these results are extremely preliminary. Similarly, the increased frequency in expression of eb3 in the treated individuals versus the control after exposure to a purifying concentration (LD$_{90}$) of naled suggests this esterase may be involved in resistance as well. However, not all esterases seem to be associated directly with the increased frequency of resistance to naled. For example, the extreme variability of eb2 in individuals from control and treated groups suggests that this band is not associated with increased resistance.

Pretreatment with naled synergizes the toxicity of resmethrin in resistant (but not susceptible) mosquitoes with elevated esterase activity, providing further evidence that esterases play a role in the observed resistance. Similarly, in a recent study, the OP chlorpyrifos was shown to synergize the toxicities of two pyrethroids (Ahmad et al., 2008). Thus, it is possible that naled is inhibiting esterases that metabolize (or sequester) resmethrin in populations of *C. quinquefasciatus* from EBR Parish. This finding may have practical application in efforts to control this deadly, disease vector. A readily available synergist, such as naled, may extend the field life of resmethrin and reduce the total amount of deleterious chemical introduced into the environment as a result of mosquito control. However, before any recommendation to use naled
as a synergist can be made, more research on the health effects in humans and other animals needs to be investigated.

Taken together, results from these assays suggest that expression of resistance mechanisms is variable, but esterases are associated with resistance to naled and resmethrin in some populations of *C. quinquefasciatus* in EBR Parish. The association between resistance and esterase activity, as measured here with a model, non-insecticide substrate was not absolute. The simplest explanation is that resistant populations are heterogeneous, and resistance is associated with esterases in some, but not all populations. A second, possible explanation is that resistance is associated with esterases, but the resistant esterase has lost (or never had) activity toward the model substrate used in these studies. This suggestion is supported by qualitative differences in banding patterns of esterases in polyacrylamide gels. Finally, co-application of naled, an esterase inhibitor, resulted in increased susceptibility to resmethrin in resistant populations with increased esterase activities and further suggest an association between esterases and resistance. As a whole, these results suggest an association between esterases and resistance; however, more work is needed before esterase-mediated enhanced metabolism can be confirmed as a major mechanism of resistance in populations of mosquitoes from EBR Parish.

As previously mentioned, the association between esterases and resistance could be strengthened by future studies. Continued monitoring of susceptibility in additional populations will provide a more accurate representation of resistance in EBR Parish. In addition, new approaches to test the association between the enzyme and insecticides are needed as well. Spectrophotometric assays using additional substrates and other inhibitors of esterases (i.e., naled, paraoxon, DEF) could better elucidate the identity and function of the esterase bands that were visualized in electrophoretic gels. Finally, molecular genetic assays to examine the identity and expression of esterases would better clarify the association between insecticide resistance
and these enzymes. Only through sampling more populations and performing more investigative assays can the association between esterase mediate enhanced metabolism and resistance to naled and resmethrin be clearly understood.

3.5 References


CHAPTER 4. CONCLUSION AND SUMMARY

By the end of World War II, synthetic insecticides had demonstrated their utility and value for managing vectors of human disease. For example, hundreds of thousands of soldiers were saved from a typhus epidemic by controlling body lice populations on soldiers in trenches (Perkins & Holochuck, 1993). In addition, use of dichlorodiphenyltrichloroethane (DDT) has saved an estimated 525 million people from malaria (Shiff, 2002). However, populations of insects develop insecticide resistance in response to the extreme selection pressure resulting from insecticide exposure. Only through monitoring susceptibility and, in resistant populations, determining the underlying mechanisms, can an integrated pest management program continually provide effective, sustained control.

Mosquito control efforts on a local level have experienced success as well. In efforts to manage populations of mosquitoes that cause discomfort and disease in humans, East Baton Rouge Mosquito Abatement and Rodent Control (EBRMARC) routinely treats populations of *Culex quinquefasciatus* with two insecticides, naled and resmethrin. The objectives of this study were to survey insecticide susceptibility in populations of *C. quinquefasciatus* collected from East Baton Rouge (EBR) Parish, to investigate the association between esterases and resistance in these populations, and to develop potential countermeasures effective against populations in which esterase-mediated enhanced metabolism is expressed.

Results from this study confirmed that populations of *C. quinquefasciatus* in EBR Parish are resistant to both naled and resmethrin. Susceptibility to resmethrin varied based on collection site; however, frequencies of resistance to naled were consistently high. Whereas these results confirm that susceptibilities to insecticides have decreased, these data do not address the efficacies of these insecticides in field situations. Resistance is inevitable in any effective control program using insecticides, and monitoring susceptibilities and developing
countermeasures to slow development of resistance are components of an effective resistance management strategy. The data do suggest, however, that EBRMARC might consider incorporating different chemistries (particularly those not containing esters).

Taken together, results from these studies suggest esterases are associated with resistance to naled and resmethrin in some populations of *C. quinquefasciatus* in EBR Parish. The association between resistance and esterase activity, as measured here with a model, non-insecticide substrate, was not absolute. The simplest explanation is that resistant populations are heterogeneous, and resistance is associated with esterases in some, but not all populations. A second possible explanation is that resistance is associated with esterases, but the resistant esterase has lost (or never had) activity toward the model substrate used in these studies. This suggestion is supported by qualitative differences in esterases as visualized in polyacrylamide gels. Finally, co-application of an esterase inhibitor (in this case, naled) increased susceptibility to resmethrin, further suggesting involvement of esterases in resistance. Taken together, these results suggest an association between esterases and resistance; however, more work is needed before esterase-mediated enhanced metabolism can be confirmed as a major mechanism of resistance in populations of mosquitoes from the parish.

The association between esterases and resistance to naled and resmethrin could be strengthened in future studies. Continued monitoring of susceptibility in additional populations will provide a more accurate representation of resistance in EBR Parish. In addition, new approaches to test the association between the enzyme and insecticides need to be used as well. Spectrophotometric assays using other substrates and inhibitors (e.g., naled, paraoxon or DEF) of esterases could better elucidate the identity and function of the esterases visualized in electrophoretic gels. Finally, molecular genetic assays to examine the identity and expression of the esterases would clarify the association insecticide resistance and esterases. Only through
sampling more populations and performing more investigative assays can the association
between esterase mediate enhanced metabolism of naled and resmethrin be clearly understood.

4.1 References

Pimentel, D. & Lehman, H. (Eds.) The Pesticide Question (pp. 47-84). New York and
London: Chapman & Hall.

Jennifer Gordon was born in Colorado Springs, Colorado, on Pike’s Peak Air Force Base and traveled around the country with her family for a few years. Eventually, her family moved back way down to Kokomo, Indiana, where she was raised until leaving for college. In 2004, Jennifer began attending classes at Purdue University. While there, she worked in the Hessian Fly Genomic's Lab for the USDA ARS. Jennifer graduated from Purdue University with her Bachelor of Science in entomology in May 2008 and accepted a job with the Indiana Department of Natural Resources as an Emerald Ash Borer surveyor. In August of 2008, she moved to Louisiana after accepting a graduate assistantship to work in the toxicology lab with Dr. Jim Ottea at Louisiana State University. Upon completion of her Master of Science in entomology in December 2010, she will begin a doctoral program at the University of Kentucky working under Drs. Ken Haynes and Mike Potter in January 2011.