Esterase Mediated Insecticide Resistance in the Southern House Mosquito, Culex quinquefasciatus

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ESTERASE MEDIATED INSECTICIDE RESISTANCE IN THE SOUTHERN HOUSE MOSQUITO, *CULEX QUINQUEFASCIATUS*

A Dissertation

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 Doctor of Philosophy in

The Department of Entomology

by

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ABSTRACT

Much is known about the development of insecticide resistance associated with targeted application against insect populations. However, off-target selection by applications of insecticides in agricultural and residential sites also impacts development of insecticide resistance and is understudied. Similarly, the impact of selecting one life stage of mosquitoes on the insecticide susceptibility of different life stages is largely unknown. The first part of this study shows that susceptibility to chlorantraniliprole, which is applied in rice and sugarcane fields in LA, decreased (5.7 to 12-fold) in populations of Culex quinquefasciatus collected from near these fields compared to a reference field strain. In addition, application of bifenthrin by commercial application on an individual residence increased the resistance frequencies to bifenthrin in five nearby residential sites, where resistance frequencies between larvae and adult were highly correlated ($R^2=0.92$) suggesting that selection of adults also confers resistance to larvae. The second part of this study focused on measurement of esterase activities associated with insecticide resistance. Most such studies have measured esterases, one of the major metabolic mechanisms of insecticide resistance, using the model substrate, α-naphthyl acetate (α-NA), which has a chemical structure vastly different than the insecticide, malathion. Therefore, the second objective of this study was to validate measurement of esterase activity using model substrates through comparison with esterase hydrolyzing malathion. These results validated the use of α-NA as a model substrate to measure esterase activity in the lab. Additionally, malathion resistance in C. quinquefasciatus was highly associated with esterase activity, both with α-NA or malathion. This shows that esterase activity is a suitable biomarker for malathion resistance in adult C. quinquefasciatus. The third part of this study has examined the association between expression of EST-3 gene encoding an esterase enzyme and malathion resistance in C.
*C. quinquefasciatus*. A positive correlation was found between malathion resistance and expression of *EST-3* in field-collected *C. quinquefasciatus*. Similarly, high expression of *EST-3* was found in individuals surviving application of a diagnostic concentration of malathion compared to individuals that was killed at this dose in both lab and field-strains of *C. quinquefasciatus*. 
CHAPTER 1. INTRODUCTION

1.1. Mosquitoes and human health

Mosquitoes vector deadly, infectious pathogens that cause high morbidity and mortality in humans and pose a serious threat to public health (WHO, 2017). These pathogens include malarial parasites (*Plasmodium* spp.) and viruses associated with Yellow Fever, West Nile, Zika, Chikungunya, and Dengue (Goddard 2018). Generally, animals such as birds, deer, horses, and sheep serve as reservoirs of these pathogens, where they are acquired by mosquitoes during blood feeding (Takken and Verhulst 2013). The infected female then transmits the deadly pathogens to humans resulting in illness and death. For malaria alone, there were 241 million cases in 2020 worldwide and 627,000 people died, mostly children under 5 (WHO, 2021). Similarly, tens of millions of cases of dengue occur each year resulting in approximately 25,000 deaths, again, mainly in children (WHO, 2017; Bhatt et al., 2015).

A common, medically important mosquito in the United States is the southern house mosquito, *Culex quinquefasciatus* (Crockett et al. 2012), which is mostly distributed in the tropical and sub-tropical regions of the world and transmits a number of arboviruses including West Nile (Goddard et al. 2002) and St. Louis Encephalitis (Monath 2021). It also transmits a filarial nematode, *Wuchereria Bancrofti*, causing lymphatic filariasis (Mak 2007), which infected 51 million people in the world in 2018 (WHO, 2021).

1.2. Management of the public health threat from mosquitoes

There are two current strategies for dealing with the public health threat from mosquitoes. One approach targets the pathogens themselves (Kim 2019) while the other seeks to break the disease cycle by targeting mosquito vectors (Wilson et al. 2020). On the one hand, drugs are used to treat illness from mosquito borne pathogens (Mirzaian et al. 2010). Historically,
treatments for malaria in different parts of the world often include chloroquine phosphate, quinine sulfate or primaquine phosphate (Mirzaian et al. 2010). More recently in 2021, the WHO has recommended a malaria vaccine (i.e., RTS, S/AS01) to treat children at high risk of malaria (Laurens 2020). As a second strategy, several measures are practiced to control mosquito populations (Beier et al. 2008). Recent development of transgenic mosquitoes through addition or deletion of genes that confer fitness costs is a new approach in managing mosquito populations (Wang et al. 2021). An example of this strategy is to introduce male sterility or cytoplasmic incompatibility such that when mating occurs non-viable offspring are produced. Similarly, Wolbachia, an intracellular reproductive parasite, has been used in suppressing mosquito populations. Wolbachia-infected male mosquitoes are released into a wild population and nonviable offspring are produced following mating with wild-type females. This strategy requires multiple, male-only releases over time to suppress mosquito populations. Another approach for modifying mosquitoes in the lab is to introduce a trait to reduce its vectorial capacity (Wang and Jacobs-Lorena 2013). These genetically modified mosquitoes in the lab are released in the field, pass the modified traits to subsequent generations, and ultimately make them less fit. With the advancement of genomic technologies such as CRISPR, the gene drive approach for modifying mosquitoes has been simplified and requires no periodic release of modified mosquitoes in the environment. Gene drive is a phenomenon where certain genetic elements are inherited in high frequency despite Mendelian law of genetic inheritance and can bring fitness costs to that organism associated with the introduced trait. Gene drives dramatically increase chances that genetic elements are passed to next generation that will allow the gene to spread rapidly and establish in a population. With the use of CRISPR, mosquitoes can be modified with a gene drive package and when it mates with non-modified mosquitoes in the
field, the offspring gets one copy of DNA from either parent (modified and non-modified). Mating activates the gene drive containing CRISPR sequences that recognizes the target gene in the opposite chromosome, which then is digested by Cas9 endonuclease. Once the gene is excised, the cell’s repair machinery is triggered, and homologous direct repair of the gene is initiated (using modified gene as a template). Ultimately, two copies of the modified gene will be present in the animal’s cells, which will be passed into next generation through the gene drive (Wang et al. 2021). This method of genetic engineering of mosquitoes is new and has not been fully implemented in the field, in part because of ethical concerns (Meghani and Boëte 2018).

At present, physical, biological, and chemical methods of suppressing mosquito populations are most commonly adopted (Rose 2001). The primary aim of physical methods is to reduce the source where mosquitoes breed without impacting the environment and other wildlife. Physical control methods disrupt the suitable breeding environment for mosquitoes. For example, reducing standing water is one of the major ways of reducing breeding site availability (AMCA, 2010). Similarly, discarding containers holding water and developing more effective drainage systems for storm water also reduces sites for mosquito breeding. Using barriers like screens, bed nets, mosquito repellents and wearing protective clothing are other examples of physical methods for reducing mosquito populations. Biological control methods aim to reduce the mosquito population with the use of plant-borne insecticides or natural enemies including mosquito predators, bacteria, and entomopathogenic fungi, and are in various stages of development (AMCA, 2010). Currently, few plant-based insecticides are in use against larval Culex, Anopheles, and Aedes species of mosquitoes (Benelli et al. 2016). Another strategy for biological control is to use predators that prey upon mosquito larvae. For example, fish, amphibians, odonate nyads, water bugs, and other mosquito species are used as predators against
larval mosquitoes (Benelli et al. 2016). Common genera of fish that have been employed in many parts of the world in controlling mosquitoes larvae include *Gambusia* and *Poecilia* (Walton 2007, Kamareddine 2012). Similarly, use of naturally occurring microbes, which are pathogenic to mosquitoes, is another strategy of biological control. *Bacillus thuringiensis var. israelensis* and *Saccharopolyspora spinosa* are the most common mosquito larvicides used in many parts of the world for controlling larval mosquitoes (Benelli et al. 2016). Finally, chemical control methods use insecticides to kill both larval and adult mosquitoes (Rose 2001).

Although there are various management techniques available for suppressing mosquito populations, use of chemicals is the most widely used, especially when there is public health threat from mosquito borne diseases such as Zika and West Nile (Rose 2001). Historically, dichlorodiphenyltrichloroethane (DDT) was one of the most successful chemical insecticides for controlling mosquito populations in many parts of the world. DDT was first registered for use against mosquitoes in 1946 (Mabaso et al. 2004), and the World Health Organization established a plan to eradicate malaria from the world by relying solely on the use of this insecticide (Nájera 1999, Nájera et al. 2011). During this time, malaria was eradicated in some countries and disease incidence was reduced dramatically in others but with growing cases of resistance and concerns about environmental and non-target effects, the use of DDT was banned in the United States in 1972. In many countries, the ban of DDT use was accompanied by a resurgence of cases of malaria (Abeyasinghe et al. 2012).

Currently, there are mainly two classes of chemicals used in suppressing adult mosquito populations. Organophosphate (e.g., malathion, naled, and temephos) and pyrethroids (e.g., bifenthrin, permethrin, sumithrin) are the two major chemical groups of insecticides used to manage populations of mosquitoes. Malathion was first registered in the United States in 1956
(Edwards 2006) and has been frequently used in suppression of adult mosquito populations. Similarly, the world is still relying on a second class of insecticides, the pyrethroids, in suppressing mosquito population, as it did in 1977 (Kupferschmidt 2016). These two groups of chemicals act at different target sites in the insects nervous system: organophosphates inhibit acetylcholinesterase in nerve cells and alter synaptic transmission (O’Brien 1976, Bajgar 2004), whereas pyrethroids prolong the closure of voltage-gated sodium channels and disrupt signal transmission (Elliott and Janes 1978, Bradberry et al. 2005).

1.3. Challenges in chemical management

Although chemical insecticides are often the cornerstone of mosquito control strategies, there are challenges associated with their use. One of the key challenges is the limited diversity of insecticidal products on the market. Most research and development in the insecticide industry has focused on agricultural chemicals, and there are few chemical insecticides (mostly organophosphates and pyrethroids) available for controlling adult mosquito populations (van den Berg et al. 2021). Another challenge in the use of chemical insecticides is their effects on non-target organisms. Many studies (Henry et al. 2012, Montagna et al. 2012, Berry et al. 2013, Palmer et al. 2013, Khan 2020) have shown impact of chemical insecticides on beneficial and non-target organisms, including recent concerns regarding effects of neo-nicotinoid insecticides on the acute, chronic, and sublethal health of pollinators (Lu et al. 2020). Similarly, there are additional concerns on the effects on pollinators of other chemicals used in suppressing adult mosquitoes. Several semi-field and lab studies have shown acute bee mortality, as well as sublethal effects, from insecticides used to suppress adult mosquito populations (Caron 1979, Pankiw and Jay 1992, Hester et al. 2001, Pokhrel et al. 2018). Effects of mosquito adulticides are not limited to pollinators but also to several families of insects, including a potential role in the decline of several rare species of lepidopterans in Florida (Calhoun et al. 2000, Mulé et al. 2017).
The other great challenge for the use of chemical insecticide is the development of insecticide resistance.

1.4. Insecticide resistance

Insecticide resistance is the inevitable outcome of the application of insecticides. Insecticide resistance results from expression of heritable traits that decrease the susceptibility of pest populations towards insecticides and often results in less than adequate control of insect pests when the insecticide is used according to the label recommendation (IRAC, 2021). Resistance is an evolutionary phenomenon in which initially rare individuals expressing resistance alleles can survive exposure to insecticide. Following frequent use of insecticides, selection occurs on such individuals: they survive and produce offspring with resistance phenotypes increasing in frequency in selected populations (Mallet 1989). Resistance to arthropods was first documented in 1914 in the San Jose scale resistant to sulphur-lime (Melander 1914). Insecticide resistance was not common before the origin of synthetic insecticides during the 1940s and 1950s (Georghiou 1990, Whalon et al. 2008, Sparks and Nauen 2015) but after widespread use of synthetic insecticides such as DDT, the cyclodienes, and organophosphates, cases of insecticide resistance grew exponentially in crop pests, as well as vectors of pathogens causing human diseases (Sparks et al. 2021).

Insecticide resistance has been a recurring challenge in vector control programs. DDT was first registered for use to control *Anopheles gambiae*, a mosquito vector of malaria, in 1946 (Mabaso et al. 2004). The widespread use of DDT as a residual house spray led to the near eradication of malaria (Mabaso et al. 2004), during a campaign that started in 1960s with the support of World Health Organization and depending solely on DDT sprays (Nájera 1999, Nájera et al. 2011). However, the goal of eradication was formally dropped because of growing
frequencies of DDT resistance in mosquito vectors (Greenwood et al. 2008). Shortly after DDT was banned in 1972, mosquito control programs shifted to a newer class of insecticide, the pyrethroids. Permethrin was used in the fibers of bed nets to protect people from mosquitoes (Snow et al. 1987); however, mosquitoes rapidly developed resistance to this chemical, which reduced the efficacy of such strategy (Ranson et al. 2011). Currently, insecticide resistance in mosquitoes is widespread: increased incidence and magnitude of resistance in mosquitoes to all major classes of insecticides have been identified across the world (Coleman et al. 2017).

1.5. Selection for insecticide resistance

The most important factor in the development of insecticide resistance is selection of insect pests through frequent use of insecticides. Selection from direct, targeted spray of insecticides on mosquito populations is common and the most studied (Crow 1957, Georghiou 1972, Liu et al. 2004, Davari et al. 2007, Cuamba et al. 2010, Edi et al. 2012, Casimiro et al. 2014). However, insecticide selection also occurs on non-targeted populations of insects from the accidental exposure to insecticide, which facilitates development of insecticide resistance. For example, irrigated agricultural fields are a favored breeding ground of mosquito populations, and these populations are often unintentionally selected with agrochemicals that may impact the insecticide susceptibility of these off-target populations, ultimately reducing the efficacy of insecticides used in mosquito control. Similarly, often intensive, application of insecticides at an individual residence is another potential source of unintentional exposure of non-targeted mosquito populations. Repeated residential use of insecticides to suppress mosquito populations not only impacts the susceptibility of targeted populations but also may influence the insecticide susceptibility of neighboring off-target mosquito populations. Further, insecticide selection occurs on different life stages of mosquitoes; however, the impact of selection of the adult on
susceptibility of larval mosquitoes (and vice versa) is largely unknown. The knowledge of stage-specific insecticide susceptibility is important for formulating life stage-specific strategies to manage insecticide resistance. The first objective of my research was to examine the impact of non-conventional selection by insecticides on susceptibility of the southern house mosquito, *C. quinquefasciatus*.

1.6. Insecticide resistance management

Insecticide resistance is an unavoidable consequence from the selection by insecticides. Thus, management strategies are essential to slow development of resistance in pest populations. Monitoring for insecticide resistance is the first component of resistance management strategies, which provides a measure of the severity of the resistance problem. Monitoring through surveillance of insecticide susceptibility involves bioassays to detect the intensity and frequency of resistance in the area. There are several types of bioassays to measure insecticide susceptibility of mosquito populations. For example, contact bioassays with discriminating concentrations of insecticides are common and easy methods to test for frequencies of insecticide resistance. In addition, topical bioassays are one of the most accurate measurements of insecticide susceptibility of populations of adult mosquito. Adult mosquitoes are topically treated with various concentrations of insecticide using a syringe fitted with repeating dispenser. Larval bioassays are conducted generally in a glass dish filled with water, where an aliquot of insecticide is added. Knowledge of insecticide resistance mechanisms are essential to develop rational, informed, and effective management strategies for insecticide resistance (Soderlund and Bloomquist 1990). Biochemical tests coupled with biological assays provide adequate sensitivity to resistance monitoring with the knowledge of intensity, frequency, and mechanism of resistance.
1.7. Mechanisms of insecticide resistance

1.7.1. Reduced cuticular penetration

Reducing the penetration of insecticides through the insect integument slows the insecticide from reaching its target site in nerve cells and is the least well understood mechanism of insecticide resistance (Oppenooorth 1984, Scott 1990). Slowing the rate of penetration of insecticide will provide more time for different detoxification enzymes to act on insecticides, and therefore minimizes the chance that insecticides reach the target site (Coleman et al. 2017). Although, expression of reduced penetration alone is less significant compared to other mechanisms, it often amplifies effects when expressed along with additional resistance mechanisms. In addition, reduced penetration can act across classes of insecticides (i.e., confer cross-resistance) at the level of the insect cuticle.

The two major ways that penetration of insecticide is reduced are by either thickening or altering the composition of cuticle (Balabanidou et al. 2018). Populations of Anopheles gambiae exhibited higher tolerance to various insecticides, which was associated with thickened cuticles in legs because of higher deposition of hydrocarbons in the epicuticle (Balabanidou et al. 2016). Other populations of Anopheles gambiae from West Africa, resistant to pyrethroids and DDT, had thicker exo-, meso,-and endocuticle that was associated with overexpression of genes for cuticular proteins (CPLCG3, CPR124, CPR127, CPR129) (Yahouédo et al. 2017). The enzyme laccase 2 is primarily involved in the altered cuticular composition leading to cuticular hardening (Balabanidou et al. 2018). Laccase 2 is an oxidase that is responsible for tanning (i.e., sclerotization and pigmentation) of insect cuticle through oxidative conjugation of quinones, which lead to the cross-linking of adjacent polypeptide chains within the cuticle (Riedel et al. 2011). Fenvalerate-resistant Culex pipens, overexpressed the CpLac2 gene compared to a susceptible strain (Pan et al. 2009). Similarly, overexpression of ATP-binding cassette (ABC)
transporters genes in insecticide-resistant pests can facilitate export of cuticular components into the cuticle. Higher expression of ABC transporter genes in the legs of resistant mosquitoes could account for elevated cuticular hydrocarbon deposits and cause reduced cuticular penetration (Pignatelli et al. 2018). In all these cases, changes in cuticle are associated with overexpression of genes encoding P450s (CYP4G16), cuticular proteins, laccase and ABC transporters.

1.7.2. Reduced target site sensitivity

Modification of insecticide target proteins such that the sensitivity to insecticides is reduced, is a major mechanism of insecticide resistance. Mutations in genes result in modified target proteins that have reduced binding affinity and efficacy of insecticides. A well-studied example of reduced target site sensitivity over the last decade is that towards pyrethroids and DDT that results from point mutations in the voltage-gated sodium channel, also known as knock down resistance (Narahashi 1988, Davies et al. 2007, Soderlund et al. 2010). Pyrethroids and DDT modify the closing of voltage-gated sodium channels (Narahashi 1988), which prolongs membrane depolarization and synaptic disturbances in the neuron. A mutation associated with knock down resistance was first detected in the pyrethroid-resistant kdr strain of house fly with the substitution of leucine to phenylalanine at position 1014 in the sixth segment of domain II of the sodium channel (Williamson et al. 1993, Knipple et al. 1994, Ingles et al. 1996, Williamson et al. 1996). The kdr mutation has been documented globally in different insect pests, including diseases vectors (Davies et al. 2007, Dong 2007, Soderlund et al. 2010, Rinkevich et al. 2013). In the mosquito, a single mutation, or combination of different mutations within the gene for the sodium channel have been shown to confer insecticide resistance (Singh et al. 2010, Srisawat et al. 2010, Jones et al. 2012). Recent studies have reported the existence of both synonymous and nonsynonymous mutations in the sodium channel of resistant mosquitoes (Li et al. 2012, Xu et
Recently, rapid methods to detect kdr-associated resistance have been developed and used in different insect pests including mosquitoes (Kulkarni et al. 2006, Hodgdon et al. 2010, Nyoni et al. 2011, Yanola et al. 2011).

Similarly, mutations in the acetylcholinesterase (AChE) gene are responsible for organophosphate and carbamate resistance (Oppenoorth 1985). This protein terminates synaptic transmission by hydrolyzing the neurotransmitter acetylcholine (Rockhold 2002). Organophosphate and carbamates inhibit AChE and prevent the breakdown of acetylcholine, culminating in over-excitation of neurons, and death by paralysis (Eldafrawi 1985). Mutations in the *ace-1* gene has been associated with resistance to OP and carbamate insecticides in mosquitoes (Weill et al. 2002, Weill et al. 2003, Alout et al. 2008), where two mutations at the active site of *ace-1* gene in mosquitoes have been reported to reduced sensitivity to both organophosphate and carbamates (Weill et al. 2002, Nabeshima et al. 2004, Liu et al. 2005).

### 1.7.3. ABC transporters

ATP-binding cassette (ABC) transporters are membrane proteins found in both prokaryotes and eukaryotes (Dermauw and Van Leeuwen 2014) and are essential to facilitate transport of a wide range of substrates across the cellular membrane through binding and hydrolysis of ATP (Merzendorfer 2014). ABC transporters in eukaryotic organisms help in export of substrates from the cytoplasm, whereas bacterial ABC transporters can also import substrates to the cytoplasm (Nwabufo 2022). These proteins mediate the transport of wide range of compounds such as sugars, amino acids, peptides, lipids, heavy metal ions, xenobiotics, and chemotherapeutic drugs across cellular membranes (Rees et al. 2009) and consists of four components: two nucleotide binding domains and 2 transmembrane domains (Dean et al. 2001, Dermauw and Van Leeuwen 2014). The transport cycle begins by binding of a compound to the
transmembrane domain, which subsequently changes the conformation of the nucleotide binding domain to facilitate ATP binding. Binding of ATP causes a major conformational change in the transmembrane domain, which rotates and open towards the outside, initiating substrate translocation. Finally, ATP is hydrolyzed to return the transporter to its original state. ABC transporters have been well-studied in conjunction with multidrug resistance in human cancer cells, where higher expression of genes encoding ABC transporters increase efflux to ending lower intracellular concentrations of chemotherapeutic drugs (Nwabufo 2022). It is assumed that insects may develop insecticide resistance using the same mechanism (Dermauw and Van Leeuwen 2014). The role of these transporters has been shown in deltamethrin-resistant cabbage looper, *Trichoplusia ni*, where a subset of ABC related transporters is associated with the increased efflux of deltamethrin (Simmons et al. 2013). In addition, a mutation in *Heliothis virescens* conferring resistance to BtCry1Ac toxin with a mechanism involving ABC transporter was found (Gahan et al. 2010). To date, there is little knowledge on the role of ABC transporters in insecticide-resistant mosquitoes. Few homologous genes from mammalian ABC transporters have been found in pyrethroid-resistant *Aedes aegypti* (Bariami et al. 2012).

1.7.4. Enhanced metabolism

Metabolic resistance is the most common and perhaps, most major mechanism of insecticide resistance (Li et al. 2007). Enzymes detoxify non-polar xenobiotics into less toxic and more polar compounds that can be rapidly eliminated from the body. These enzymes have substrate specificities including xenobiotics, steroids, bile acids, fatty acids, hydrocarbons, drugs, and allelochemicals (Brattsten 1988, Nebert and Russell 2002, Francis et al. 2005). Resistant insects detoxify the insecticides rapidly and efficiently because of qualitative or quantitative changes in these enzymes (Hemingway 2000, Li et al. 2007, Panini et al. 2016). Qualitative
changes in enzymes metabolize insecticides with higher catalytic rate, whereas quantitative changes produce greater quantities of enzymes that can metabolize or sequester insecticides (Hemingway 2000). Detoxication is broadly categorized into two phases: phase I (primary) involves hydrolysis or oxidation and phase II (secondary) enzymes conjugate the metabolized products from phase I with endogenous compounds such as glutathione, which facilitates excretion from the body (Iyanagi 2007). Detoxication strategies in resistant insects rely on metabolism of insecticides through enzymatic catalysis but also involve sequestration that prevents or retards the movement of insecticide to their target sites. The major detoxifying enzymes associated with insecticide resistance in insect pests are transcribed by members of large multigene families encoding cytochrome P450 monooxygenases, glutathione-S transferases, and esterases.

**Monooxygenase.** Monooxygenases are phase I enzymes involved in the detoxication of a wide range of exogenous (e.g., plant allelochemicals and insecticides) and endogenous (e.g., hormones, pheromones, fatty acids) compounds (Feyereisen 1999, Scott 1999). Cytochrome P450 monooxygenases are membrane bound, predominantly in the endoplasmic reticulum, and belong to a group of heme thiolate proteins (Slaughter and Edwards 1995). These enzymes are involved in the oxido-reduction process, where reduction of oxygen occurs to form water and an oxidized product that is most often less toxic (Guengerich 2001, Feyereisen 2005). These enzymes catalyze a large number of different reactions such as epoxidation, hydroxylation, N-dealkylation, O-dealkylation or desulphurization (Oppenoorth 1985, Soderlund and Bloomquist 1990). CYP gene families are large and varied across different insects (36 CYP genes in body louse to 200 CYP genes in Culicine mosquitoes) (Lee et al. 2010, Yang and Liu 2011), and cases of insecticide resistance against broad classes of insecticides have been associated with increased
levels of P450s (Scott 1999, Daborn et al. 2002, Daborn and Le Goff 2004). Many cases of P450-associated insecticide resistance were due to gene upregulation through regulatory elements (Feyereisen 2005). However, gene amplification or qualitative changes in CYP genes or resistant insect pest have also been reported (Amichot et al. 2004, Wondji et al. 2009, Puinean et al. 2010). Overexpression of multiple P450 genes have been observed in many resistant insect pests including mosquitoes (Festucci-Buselli et al. 2005, Zhu et al. 2008, Zhu and Liu 2008, Liu et al. 2011). For example, the expression profile of 204 P450 genes in resistant *Culex quinquefasciatus* reflected multiple, up-regulated P450 genes (Yang and Liu 2011).

**Glutathione-S-transferase.** Glutathione-S-transferases are a large family of phase II detoxification enzymes involved in the conjugation of reduced glutathione (GSH) with electrophilic substrates such as insecticides, making more water soluble products, which are most often nontoxic (Pavlidi et al. 2018). Like P450s, GSTs have a broad range of substrate specificities from endogenous to xenobiotic compounds and are also associated with oxidative stress (Ketterman et al. 2011). These enzymes are mostly involved in organophosphate and pyrethroid resistance in different insect pests (Clark 1989, Pavlidi et al. 2018). In addition, GST has been reported in the involvement of DDT resistance in house fly and in mosquitoes (Prapanthadara et al. 1993, Ahmed et al. 1998). GSTs are constitutively over-expressed in DDT-resistant *Aedes aegypti* and *Anopheles gambiae* (David et al. 2005, Strode et al. 2008).

**Esterases.** Esterases hydrolyze ester-containing chemicals into an alcohol and acid by the addition of water to produce products that are more water soluble and less toxic. Esterases have a wide array of substrates including carboxylic, thio-, phosphoro-, and other esters (Oakeshott et al. 2010). Esterases are broadly classified into three different classes (A-, B-, and C-type esterases) based on their interaction with OP molecules such as paraoxon (Aldridge 1973).
Esterases that are capable of hydrolyzing OP compounds are called A-type esterases, whereas esterases inhibited by OP compounds are called B-type esterases. Esterases that do not interact with OP compounds are called C-type esterases. A-type esterases mostly have a cysteine residue in their active site while B-type esterases have a serine residue in their active site. The most well-studied insect esterases are B-type esterases. In addition, there are different eukaryotic esterases that have been classified based on interactions with three classes of inhibitors (i.e., OPs, sulfhydryl reagents, and the carbamate eserine sulfate; (Holmes and Masters 1967, Coates et al. 1975, Healy et al. 1991). For example, AChE is inhibited by OPs and eserine sulfate, whereas carboxylesterases are only inhibited by OPs. Similarly, arylesterases are inhibited by sulfhydryl reagents whereas acetyesterase are unaffected by any of these compounds. In addition to this general classification, different nomenclatures have been used to define the type of esterase in particular species. For Culex mosquitoes, esterases are classified based on their substrate preference for, α- and β-naphthyl acetates and their electrophoretic mobility. The esterases that preferentially hydrolyze α- and β- naphthyl acetates are called Estα and Estβ esterases, respectively (Hemingway and Karunaratne 1998).

Although esterases exist as large families with different substrate specificities, mechanisms of most of the esterases are similar. The B-type esterase contain a serine -histidine-glutamic acid catalytic triad where oxygen of the serine residue makes a nucleophilic attack on the carbonyl atom of the substrate forming a acylated enzyme which is followed by hydrolysis of the acylated enzyme through the addition of water, releasing the free enzyme and acid product of the reaction (Sogorb and Vilanova 2002, Testa and Kraemer 2007, Russell et al. 2011). In the case of insecticides such as OPs and carbamates, the acylated enzyme is formed quickly but its
rate of hydrolysis is retarded (Walker and Mackness 1987). Thus, the process of formation of free enzyme from acyl-enzyme is extremely (OPs) or very (carbamates) slow and rate limiting.

Esterases have been associated with insecticide resistance in many insect pests including mosquitoes. Esterase-mediated insecticide resistance is generally due to either expression of enzymes with higher catalytic activity or sequestration or hydrolysis of insecticides through elevated titers of wild-type enzymes (Hemingway 2000). Elevated level of enzymes sequesters ester insecticides, which prevents them from interacting with their target sites. Enhanced expression of esterases in resistant insect pests can involve gene amplification or upregulation (Li et al. 2007). For example, in the green peach aphid Myzus persicae, overproduction of esterase due to gene amplification contributed to the development of insecticide resistance (Field et al. 1988). Similarly, an amplified esterase has been associated with insecticide resistance in C. quinquefasciatus (Hemingway et al. 2004) and the brown plant hopper Nilaparvata lugens (Small and Hemingway 2000). However, in some insect pests such as Aphis gossypi (Cao et al. 2008) and Bemisia tabaci (Alon et al. 2008), the increased expression of esterases in resistant strains was due to increased transcription resulting from upregulation of corresponding genes. Esterase-mediated resistance also results from changes in the structure of genes that enhance the capacity to metabolize insecticides. For example, in malathion-resistant house fly (Oppenoorth and van Asperen 1960) and sheep blow fly (Campbell et al. 1998), a mutation decreased the catalytic efficiency of the mutant esterase to alpha naphthyl acetate (α-NA) but increased hydrolysis of malathion. This hydrolysis of insecticide with concurrent decrease in hydrolysis of model substrates is the basis of the “mutant ali-esterase” theory, where the substrate specificity of mutant esterases changes from model substrate to insecticide.
Despite the variable relationship between hydrolysis of model substrates and insecticides, most of the esterase mediated insecticide resistance has been measured in the lab using model substrates (Lewis and Madge 1984, Grant et al. 1989, DeSilva et al. 1997, Baker et al. 1998, Gordon and Ottea 2012), using quick and easy spectrophotometric assays. However, as mentioned above, a variable relationship between esterases hydrolyzing malathion and model substrates has been found in different malathion-resistant insect pests (Oppenoorth and van Asperen 1960, Hemingway 1985, Hughes and Raftos 1985, Ziegler et al. 1987, Baker et al. 1998, Gao et al. 2006). In addition, elevated malathion carboxylesterase activity with no change in esterase activity hydrolyzing model substrates was found in Anopheles and Culex tarsalis mosquitoes, but no detectable malathion carboxylesterase activity was found in malathion resistant Culex quinquefasciatus (Whyard et al. 1995, Karunaratne and Hemingway 2001). Thus, the biochemistries of carboxylesterases hydrolyzing malathion and model substrates are not fully resolved and are variable among different species of insects. Additionally, the chemical structures of model substrate and the insecticide malathion are completely different and difference in substrate preference might be expected. Therefore, it is prudent to validate use of model substrates for the measurement of resistance-associated esterases. My second objective was to validate the measurement of esterase activity using α-NA in malathion-resistant C. quinquefasciatus.

Esterases of insects are broadly divided into 14 clades depending on catalytic capability and cellular or subcellular localization (Oakeshott et al. 1999, Oakeshott et al. 2010). Catalytic capabilities are based on the consensus sequence in the catalytic triad. Broadly, insect esterases can be divided into three groups that include a total 14 of clades. One group consists of secreted proteins, which are membrane-associated and have mostly neurodevelopmental function. The
second group is almost all secreted but not membrane associated and are catalytically competent. For example, insect juvenile hormone esterase falls in this group. The third group has a range of cellular or subcellular localization such as microsomes, cytosol, mitochondria, and have dietary detoxification functions.

Esterases of mosquitoes studied to date fall under the third group described above. In *C. quinquefasciatus*, there are many esterase genes with both known and unknown functions (Yan et al. 2012). The best studied and most important in the case of insecticide resistance in *C. quinquefasciatus* are EST-3 and EST-2 genes, also called estα and estβ based on substrate preference for α and β- naphthyl acetate, respectively (Wirth et al. 1990, Ketterman et al. 1993, Vaughan et al. 1997, Talipouo et al. 2021, Vivekanandhan et al. 2021). These two genes are arranged head-to-head with short intergenic sequence around 1.7 kb, and are overexpressed in most cases of organophosphate resistance in *C. quinquefasciatus* due to either gene amplification or mutations in regulatory elements. (Peiris and Hemingway 1993, Lee et al. 2012, Kothera et al. 2019, Talipouo et al. 2021). Most studies (Liu et al. 2007, Yang and Liu 2011, Reid et al. 2012, Gong et al. 2022) have focused on characterizing expression of these genes in insect pests, without clearly establishing the role in the development of insecticide resistance. Therefore, my third objective was to examine the expression profile of EST-2 and EST-3 genes in lab and field strains of *C. quinquefasciatus*, with variable levels of resistance to malathion.

1.8. Specific objectives

1. To measure the impact of non-conventional selection by insecticides on susceptibility of the southern house mosquito, *Culex quinquefasciatus*

2. To optimize and validate the measurement of esterase activity using a model substate in field-collected populations of *C. quinquefasciatus*
3. To examine the expression of candidate genes encoding major detoxifying enzyme activities in malathion-resistant *C. quinquefasciatus*
2.1. Introduction

Development of resistance from frequent use of chemical insecticides has compromised efforts to suppress mosquito populations. Use of insecticides is one of the most common and effective strategies for abatement, which is especially important when there is a high risk of mosquito nuisance and associated diseases (Rose 2001). However, application of insecticides selects pest populations by allowing initially rare individuals that express resistance phenotypes to survive exposure and pass their resistance traits to offspring (Mallet 1989, McKenzie and Batterham 1994). Thus, insecticide resistance results from heritable traits that decrease the susceptibility of insect populations towards insecticides and is a direct consequence of exposure to insecticides in the field.

Whereas selection from direct, targeted spray of insecticides on insect populations is common and well-studied (Crow 1957, Georghiou 1972, Liu et al. 2004, Davari et al. 2007, Cuamba et al. 2010, Edi et al. 2012, Casimiro et al. 2014), selection on non-targeted insect populations from unintentional exposure also impacts development of insecticide resistance and is understudied. For example, irrigated agricultural fields are a favored breeding ground of mosquitoes, which may be unintentionally selected with agrochemicals. This off-target exposure may impact the insecticide susceptibility of these populations, ultimately reducing the efficacy of insecticides used in mosquito control. Earlier studies have reported development of resistance against different insecticides in such non-targeted populations of the black fly, Simulium slossonae (Montagna et al. 2012) and house fly, Musca domestica (Khan 2020). In addition, the impact of indirect exposure to organochlorine and pyrethroid insecticides on insecticide
susceptibility in non-targeted populations of *Anopheles gambiae* from agricultural fields has been described (Diabate et al. 2002, Chouaibou et al. 2008, Yadouleton et al. 2009). Similarly, often intensive, residential spray of insecticides is another potential source of unintentional exposure to non-targeted mosquito populations. Repeated residential use of insecticides to suppress mosquitoes not only impacts the susceptibility of targeted populations but also may influence the insecticide susceptibility of neighboring off-target populations. To our knowledge, no previous studies have examined the effect of insecticide sprays at individual residences by commercial pest control applicators on the insecticide susceptibility of neighborhood mosquito populations.

Finally, few studies have examined the impact of selection on one life stage of mosquitoes on susceptibility in other life stages. Both larvicides and adulticides are used in mosquito control programs, providing intense selection on both life stages. However, the impact of selection of the adult on susceptibility of larval mosquitoes (and vice versa) is largely unknown. The life stage specific sensitivity to cyhalothrin has been measured in the house fly (Fu-xing et al. 2002), where larvae were over 100-fold more resistant than adults. In contrast, Brewer et al. (1990) found that larval and adult susceptibilities to fenvalerate and methomyl were correlated in field-collected beet armyworms, *Spodoptera exigua*. In mosquitoes, differential expression of genes encoding different detoxification enzymes was found between larval and adult *Anopheles gambiae* (Strode et al. 2006), but the impact of this difference on insecticide susceptibility was not measured.

The knowledge of stage-specific susceptibility towards insecticides is critical for formulating life stage-specific strategies to manage insecticide resistance. Adult (but not larval) populations of mosquitoes are generally selected with ester-containing insecticides such as
organophosphates and pyrethroids, and esterases are often associated with resistance to these insecticides in adult insects (Hemingway and Ranson 2000). Esterases are a group of major detoxifying enzymes that hydrolyze organophosphates and pyrethroids into less-toxic products and have been studied extensively as a major mechanism of insecticide resistance (Hemingway and Karunaratne 1998, Hemingway 2000, Xu et al. 2005, Gordon and Ottea 2012, Gong et al. 2022).

In this study, the role of off-target selection from the application of an agrochemical (i.e., chlorantraniliprole) on susceptibility of *C. quinquefasciatus* was examined. Chlorantraniliprole and malathion susceptibilities of *C. quinquefasciatus* collected from rice and sugarcane fields were measured. In addition, we examined the impact of insecticide application at an individual residence on resistance frequencies of mosquitoes collected from other, unsprayed residences along a transect in the same neighborhood. Finally, we measured and compared bifenthrin susceptibilities in larval and adult mosquitoes collected at the residential sites, and measured contribution of esterase activities as a mechanism of insecticide resistance in the two life stages.

**2.2. Materials and methods**

**2.2.1. Chemicals**

Malathion (99.5%), bifenthrin (99.5%), and chlorantraniliprole (99.5%) were purchased from Chem Services (West Chester, PA). 1-Naphthyl acetate (α-NA; ≥98%), Fast Blue B salt (approx. 95 %), Brilliant Blue G-25 (ultra-pure), sodium phosphate monobasic monohydrate (≥98%), sodium phosphate dibasic heptahydrate (98%), sodium dodecyl sulfate (SDS; 99%), acetone (99.5%) and phosphoric acid (ACS grade) were purchased from Millipore Sigma (Burlington, MA). Hydrochloric acid (99.7%), phosphoric acid (85%), potassium chloride (ACS grade) and sodium hydroxide (ACS grade) were purchased from Fisher Scientific (Kansas City,
MO). Bovine serum albumin (Biotechnology grade) was purchased from Amresco (Solon, OH). Absolute ethanol (ACS/UPS grade) was purchased from Pharmco-Aaper (Brookfield, CT). Yeast powder was purchased from Solgar (Leonia, NJ) and beef liver powder was purchased from Now Foods (Bloomingdale, IL).

2.2.2. Insects

Sebring-S (SEB-S), a reference-susceptible lab strain of *C. quinquefasciatus*, with no known history of exposure to insecticides, was originally colonized at the United States Department of Agriculture Research Station in Gainesville, FL and was provided by the Medical Entomology Lab at LSU. A reference-susceptible field strain of *C. quinquefasciatus* was collected near the LSU Lake (Table 2.1; LSU LAKE-S) with no recorded history of exposure to insecticides. Field populations of *C. quinquefasciatus* collected from agricultural sites where chlorantraniliprole was applied in fields of rice (as a seed treatment; RICE) and sugarcane (as a foliar spray; CANE-1 and CANE-2) against stem boring pests. Field populations of *C. quinquefasciatus* were also collected from residential sites along a linear transect on the same street. The sprayed residential site (0-0) was treated repeatedly with bifenthrin against mosquito populations by a commercial pest applicator (Table 2.1). A total of five non-sprayed residential sites were also sampled: two located north (i.e., N-1, N-2) and three located south (i.e., S-1, S-2, S-3) of the sprayed residential site (0-0). The total distance between the sites located from the far north to the far south of the residential street was 343.13 meters.

Egg rafts were collected from all field sites using black plastic containers containing yeast powder (2 gm) and aged, distilled water (4 L). Field-collected populations of *C. quinquefasciatus* were used in the same generation in which they were collected. Larval and adult *C. quinquefasciatus* were reared under the condition of constant photoperiod
Table 2.1. Mosquito populations used in the study.

<table>
<thead>
<tr>
<th>Mosquito population</th>
<th>Collection site</th>
<th>GPS coordinates</th>
<th>Parishes</th>
<th>Collection date</th>
<th>Sprayed chemicals</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEB-S</td>
<td>Laboratory</td>
<td></td>
<td></td>
<td></td>
<td>Unsprayed</td>
</tr>
<tr>
<td>LSU LAKE-S</td>
<td>Residential</td>
<td>30°24'33&quot; N 91°10'W</td>
<td>East Baton Rouge</td>
<td>2021-August and November 2022-June</td>
<td>Unsprayed</td>
</tr>
<tr>
<td>CANE-1</td>
<td>Sugarcane field</td>
<td>30°27'59&quot; N 91°17'44&quot;W</td>
<td>West Baton Rouge</td>
<td>2020-August</td>
<td>Chlorantraniliprole</td>
</tr>
<tr>
<td>CANE-2</td>
<td>Sugarcane field</td>
<td>30°16'4&quot; N 91°6'29&quot;W</td>
<td>Iberville</td>
<td>2021-August</td>
<td>Chlorantraniliprole</td>
</tr>
<tr>
<td>RICE</td>
<td>Rice field</td>
<td>30°14'23&quot; N 92°20'45&quot;W</td>
<td>Acadia</td>
<td>2021-August</td>
<td>Chlorantraniliprole</td>
</tr>
<tr>
<td>0-0</td>
<td>Residential</td>
<td>30°25'56&quot; N 91°9'52&quot;W</td>
<td>East Baton Rouge</td>
<td>2021-August and November 2022-June</td>
<td>Bifenthrin</td>
</tr>
<tr>
<td>N-1</td>
<td>Residential</td>
<td>30°25'59&quot; N 91°9'52&quot;W</td>
<td>East Baton Rouge</td>
<td>2021-August and November 2022-June</td>
<td>Unsprayed</td>
</tr>
<tr>
<td>N-2</td>
<td>Residential</td>
<td>30°26'1&quot; N 91°9'52&quot;W</td>
<td>East Baton Rouge</td>
<td>2021-August and November 2022-June</td>
<td>Unsprayed</td>
</tr>
<tr>
<td>S-1</td>
<td>Residential</td>
<td>30°25'54&quot; N 91°9'52&quot;W</td>
<td>East Baton Rouge</td>
<td>2021-August and November 2022-June</td>
<td>Unsprayed</td>
</tr>
<tr>
<td>S-2</td>
<td>Residential</td>
<td>30°25'52&quot; N 91°9'53&quot;W</td>
<td>East Baton Rouge</td>
<td>2021-August and November 2022-June</td>
<td>Unsprayed</td>
</tr>
<tr>
<td>S-3</td>
<td>Residential</td>
<td>30°25'49&quot; N 91°9'53&quot;W</td>
<td>East Baton Rouge</td>
<td>2021-August and November 2022-June</td>
<td>Unsprayed</td>
</tr>
</tbody>
</table>
(14 h light:10 h dark) and humidity (70%). Larvae were fed daily a mixture of liver powder and yeast (1:1) and adults were provided with 10% sugar solution. Once per generation, adult females (7-10 days old) were fed defibrinated chicken blood (Rockland Immunochemical, Pottstown, PA) using a membrane feeding system (Hemotek, Blackburn, England).

2.2.3. Biological assays

Adult susceptibility of *C. quinquefasciatus* to insecticides was examined using a topical bioassay. Adult females (3-7 days old) were anesthetized with CO₂ for less than 1 min then treated with 0.5 µl of acetone solutions containing malathion or bifenthrin on the thoracic dorsum using a syringe fitted with a repeating dispenser (Hamilton, Reno, NV). A full range of doses were used to measured malathion susceptibility in adults whereas a diagnostic concentration was calculated and used to measure bifenthrin susceptibility in adults (i.e., 10XLD₅₀ obtained from log dose probit line using the SEB-S strain of mosquito). The control group of insects was treated with acetone only. Treated adult females were then placed in waxed paper cups (270 ml capacity) fitted with a fine mesh top and fed by placing a 10% sucrose-soaked cotton ball on top of the mesh. Treatment cups were incubated as described above. Mortality was recorded 18 hours after treatment and was defined by an inability of treated insects to right themselves after being flipped onto their dorsa. Larval susceptibility to insecticides was examined in a Pyrex glass dish filled with 100 ml of aged water containing early fourth instars, into which a one ml aliquot of malathion, bifenthrin, or chlorantraniliprole was added. A full range of concentrations was used to measure malathion (0.003-0.12 ppm) and chlorantraniliprole (0.4-15 ppm) susceptibilities in larvae of *C. quinquefasciatus*, whereas a diagnostic concentration (i.e., 10XLC₅₀ obtained from log dosage probit line using the SEB-S strain of mosquito) was used to measure bifenthrin susceptibility in larvae. Control and treated mosquitoes were held in
environmental chambers as described above. Mortality was recorded 18 hrs. after treatment and was defined by an inability to move after being poked by a pipette tip. Control mortality for all biological assays was below 5% in all experiments and was corrected using Abbott’s formula (Abbott 1925).

2.2.4. Measurement of Esterase Activity

Esterase activity towards α-NA was measured using the colorimetric assay described by Gomori (1953) as modified by van Asperen (1962) and Grant et al., (1989) in polystyrene 96-well flat bottom microplates (Costar, Cambridge, MA). Mosquitoes were homogenized in 1.15% KCl using 10 strokes of an all-glass mortar and pestle then centrifuged at 4°C for 10 mins at 15,000 g. The supernatant from this spin was used as an enzyme source immediately after preparation. A stock solution of α-NA (30 mM in acetone) was diluted 100-fold with buffer (0.1 M sodium phosphate, pH 7.4) and reactions were started by the addition of 200 µl of this solution (0.22 mM final concentration) to 20 µl of either enzyme homogenate (12-15 µg protein) or buffer (control). Reactions were stopped after 10 mins by addition of 50 µl Fast Blue B dye (2.18 mM final concentration). Optical densities of reactions were measured at 570 nm using a Thermomax microplate reader (Molecular Devices, Palo Alto, CA), and converted to µmol/min using an experimentally derived extinction coefficient for α-naphthol (2.6 µM⁻¹ 270 µl). Protein concentrations were measured using the method of Bradford (1979) with bovine serum albumin as the standard.

2.2.5. Statistics

All statistical analyses were conducted in R (Team 2013a). Data from bioassays were subjected to probit analysis using the function LC_probit from the package ecotox and resulting LD₅₀ values were used to compare insecticide susceptibilities to malathion and
chlorantraniliprole. In addition, percent mortalities following exposure to diagnostic concentrations of bifenthrin were compared among mosquitoes collected from sprayed and non-sprayed residential sites using the analysis of variance (aov) function. Tukey’s multiple pairwise comparison (Tukey HSD function; P<0.05) was made to compare resistance frequencies among different residential sites. Linear regression (lm function) was used to determine relationships between esterase activities and percent mortality resulting from exposure to diagnostic concentrations.

2.3. Results

2.3.1. Susceptibilities of C. quinquefasciatus collected from agricultural sites

The LC$_{50}$ values for chlorantraniliprole calculated for larvae collected from three agricultural sites were significantly higher than those of the reference susceptible-laboratory (SEB-S) and -field populations (LSU LAKE-S; Table 2.2). Resistance ratios measured were highest in RICE (12-fold) and lowest in CANE-2 (5.7-fold). Resistance in mosquito populations collected from the three agricultural sites was lower to malathion and ranged from 1.24-to 3.60-fold (larvae) and 1.99-to 2.80-fold (adults). The resistance ratios measured with larvae were minimal for both chlorantraniliprole (2.70-fold) and malathion (2.00-fold) at the field-reference site. Similarly, for adults resistance ratio of malathion was 1.40-fold at the field-reference site.
Table 2.2. Chlorantraniliprole and malathion susceptibilities of *C. quinquefasciatus*.

<table>
<thead>
<tr>
<th>Insecticides</th>
<th>Populations</th>
<th>Larvae</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LC₅₀ (95% CI)¹</td>
<td>RR²</td>
</tr>
<tr>
<td>Chlorantraniliprole</td>
<td>SEB-S</td>
<td>0.57 (0.50-0.63)¹⁰</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>LSU-LAKE</td>
<td>1.59 (1.30-1.92)¹³</td>
<td>2.70</td>
</tr>
<tr>
<td></td>
<td>CANE-1</td>
<td>4.46 (3.68-5.18)¹⁴</td>
<td>7.80</td>
</tr>
<tr>
<td></td>
<td>CANE-2</td>
<td>3.25 (2.40-4.20)¹⁵</td>
<td>5.70</td>
</tr>
<tr>
<td></td>
<td>RICE</td>
<td>6.90 (5.60-9.20)¹⁶</td>
<td>12.00</td>
</tr>
<tr>
<td>Malathion</td>
<td>SEB-S</td>
<td>0.02 (0.01-0.02)¹⁰</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>LSU-LAKE</td>
<td>0.03 (0.02-0.04)¹³</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>CANE-1</td>
<td>0.06 (0.05-0.07)¹⁵</td>
<td>1.24</td>
</tr>
<tr>
<td></td>
<td>CANE-2</td>
<td>0.05 (0.04-0.07)¹⁵</td>
<td>3.40</td>
</tr>
<tr>
<td></td>
<td>RICE</td>
<td>0.06 (0.05-0.07)¹⁷</td>
<td>3.60</td>
</tr>
</tbody>
</table>

¹ ppm of insecticides (95% Confidence Interval). In comparisons within life stage, values followed by the same letter are not significantly different (P>0.05)
² RR= LD₅₀ or LC₅₀ of field-collected/ LD₅₀ or LC₅₀ of Sebring-S
³ ng of malathion (95% Confidence Interval). In comparisons within life stage, values followed by the same letter are not significantly different (P>0.05)
⁴ ND = not determined
2.3.2. Effect of residential sprays on susceptibility of *C. quinquefasciatus*

There was an overall decrease in bifenthrin susceptibility in all residential sites compared to the susceptible laboratory (SEB-S) and reference-field populations (LSU LAKE-S) of *C. quinquefasciatus* for both life stages (Fig. 2.1). Percent mortalities from diagnostic concentrations of bifenthrin in larvae and adults collected from all the residential sites were significantly lower (P<0.05) than either SEB-S or LSU LAKE-S strains. Additionally, bifenthrin susceptibility in larvae and adults from all the residential sites were highly correlated (R²=0.92, Fig. 2.1; inset). There was no clear trend between bifenthrin susceptibility and distance from the sprayed site (0-0).

Figure 2.1. Bifenthrin susceptibility in larval and adult populations of *C. quinquefasciatus* collected from residential sites. Mortality was measured following treatment with 10 times the LC₅₀ (larvae) or LD₅₀ (adult) of bifenthrin calculated from assays with SEB-S. Inset: Larval vs-adult mortality. Bars represents average percent mortality (±SD) from 3 different collection of *C. quinquefasciatus*
However, there were differences in insecticide susceptibility in residential sites in different seasons (Fig. 2.2): bifenthrin susceptibilities of adult populations from all residential sites were lower in August 2021 (immediately following insecticide application) than in November 2021 or June 2022. In August 2021, mortality following application of a diagnostic concentration of bifenthrin (10XLC$_{50}$) at the sprayed site was only 8% and was significantly lower than three non-sprayed residential sites (N-1, S-1, and S-3). However, the average percent mortalities of sprayed and non-sprayed residential sites (N-2, and S-2) were not significantly different (P>0.05).

Figure 2.2. Bifenthrin susceptibility in adult *C. quinquefasciatus* collected from residential sites following treatment with 10 times the LD$_{50}$ of bifenthrin calculated from assays with SEB-S. Bars represents average percent mortality ($\pm$ SD) from 3 different collections of *C. quinquefasciatus*
2.3.3. Esterase activities of *C. quinquefasciatus* from residential sites

Esterase activities were moderately correlated with bifenthrin susceptibilities in both larval (R²=0.40) and adult (R²=0.52) populations of *C. quinquefasciatus* collected from residential sites (Fig. 2.3). Furthermore, esterase activities of larvae were correlated with those of adults (R²=0.75; Fig. 2.3, inset).

![Figure 2.3. Esterase activities and bifenthrin susceptibilities of larval (open circle) and adult (closed circle) populations of *C. quinquefasciatus* collected from residential sites. Mortality was measured following treatment with 10 times the LC50 (larvae) or LD50 (adult) of bifenthrin as calculated from assays with SEB-S. Inset: Esterase activities in larvae vs-adults.](image)

**Mortality from Diagnostic Concentration (percentage)**

2.4. Discussion

Insecticide resistance is widespread and well-studied in populations of mosquitoes (Liu 2015, Coleman et al. 2017). While most studies describe effects on susceptibility in populations of mosquito targeted directly by insecticide application, less traditional sources of selection, such
as insecticide run-off from agricultural fields, have been less well-studied despite potential impacts on susceptibility of mosquito populations in areas adjacent to sprayed fields. In the current study, susceptibility to chlorantraniliprole (which is not used in mosquito abatement) was reduced in populations adjacent to both rice and sugarcane fields, where this insecticide is applied to manage stem boring pests. Chlorantraniliprole susceptibility in the population collected from the rice field was lower compared to that in sugarcane, a possible reflection of different degrees of selection resulting from different methods of chlorantraniliprole application. In Louisiana sugarcane fields, chlorantraniliprole is generally used as a foliar spray (Wilson et al. 2022), whereas in rice fields, it is used as a seed treatment (Stout et al. 2011, Sidhu et al. 2014), which generally increases intensity of selection by chlorantraniliprole in larvae residing in the aquatic environment of rice fields. Water management practices in the rice field can readily result in the leaching of seed treatments into nearby water sources (Gupta et al. 2008, Vela et al. 2017), which increases exposure of invertebrate communities in the aquatic environment. In previous studies, populations of mosquitoes in agricultural fields have reduced susceptibilities to DDT and deltamethrin (Overgaard 2006, Ranson et al. 2009, Yadouleton et al. 2011, Fodjo et al. 2018). As expected, malathion resistance in both larval and adult *C. quinquefasciatus* collected from agricultural sites was minimal, as malathion is not used in agricultural fields and selection pressure is low.

Insecticide application at individual residences is another non-traditional source of selection that impacts susceptibility of non-target mosquito populations. In this study, application of bifenthrin at an individual residential site by a commercial pest applicator affected the susceptibility of *C. quinquefasciatus* throughout the neighborhood. Resistance frequencies of bifenthrin in both larval and adult *C. quinquefasciatus* at all residential sites were significantly
higher compared to the reference -field (LSU LAKE-S) site. There was no clear pattern between resistance frequencies and distance from the sprayed site. However, resistance frequencies of bifenthrin varied in different seasons of the year and were lower in adult populations collected in November 2021 and June 2022 compared to August 2021, which might be due to reduced frequencies of bifenthrin application in November 2021 and June 2022. Frequency of bifenthrin resistance at the sprayed site was significantly higher than three unsprayed residential sites in summer collection (August 2021) but lower in N-2 and S-2. There are several factors that affect exposure of populations of mosquitoes to applied insecticides such as wind speed and direction, spray methods and distance from point of application. (Schleier III et al. 2012, Rinkevich et al. 2017, Desmarteau et al. 2020). In a semi-field study, (Rinkevich et al. 2017) suggest that distance is one of the most important environmental factors that effect exposure and selection of mosquitoes and honey bees with different groups of insecticide used in mosquito abatement programs. Similarly, genetic mixing among populations of *C. quinquefasciatus* at the sampled residential sites might have contributed to variable frequencies of bifenthrin resistance in unsprayed residential sites (Service 1997). Alternatively, it is possible that the same population of *C. quinquefasciatus* was being sampled from all six residential sites.

An additional, important factor that contributes to the chemical management of insects is the impact of selection on different life stages of insect pests. In the current study, larval and adult susceptibilities to bifenthrin were correlated. Because bifenthrin is generally used against adult mosquitoes in abatement programs (Hougard et al. 2002, Qualls et al. 2012), selection is most intense on this life stage. However, bifenthrin susceptibility in larval populations was decreased as well and was correlated with bifenthrin susceptibility in adults.
Esterases hydrolyze ester-containing insecticides (including bifenthrin) and have been shown in previous studies to be associated with insecticide resistance (Raymond et al. 1993, Rider et al. 1998, Jackson et al. 2013b, Wei et al. 2020). In the current study, esterase activities were moderately correlated with bifenthrin susceptibilities in both larval and adult population of *C. quinquefasciatus* (R²=0.4 and 0.52 for larvae and adults, respectively), which suggests involvement of other resistance mechanisms such as altered target sites (Lopez-Monroy et al. 2018) or enhanced metabolism by other enzymes (Riveron et al. 2013). It should be noted that target sites differed among the insecticides studied here: the ryanodine receptor for chlorantraniliprole, acetylcholinesterase for malathion, and voltage-sensitive sodium channel for bifenthrin. In addition, the contribution of other detoxifying enzyme such as P450s or glutathione-S-transferase was not studied and cannot be excluded. Finally, esterase activities in larvae and adults were correlated suggesting that expression of the esterase gene(s) was not life stage specific.

In conclusion, non-conventional sources of selection impact the development of insecticide resistance on non-targeted pest populations. Similarly, in the populations studied here, selection on adult *C. quinquefasciatus* affects insecticide susceptibility of the larval stage. Results from this study may be useful for developing management strategies for population of *C. quinquefasciatus*. 
CHAPTER 3. EXPRESSION OF ESTERASE ACTIVITIES TOWARDS α-NAPHTHYL ACETATE AND MALATHION IN MALATHION RESISTANT CULEX QUINQUEFASCIATUS

3.1. Introduction

Insecticide resistance is a major challenge to the use of chemical insecticides to control insect pests. Resistance is an unavoidable outcome of application of insecticides and a serious issue in global pest management, affecting all the major insect pests and classes of insecticides (Sparks et al. 2021). Insecticide resistance in mosquito populations was first observed following the extensive use of DDT during malaria eradication campaigns that began in the 1940s, when resistance was selected in Anopheline species that transmit malaria pathogens (Hemingway et al. 2002). Currently, insecticide resistance is widespread: increased incidence and magnitude of resistance in mosquitoes to all major classes of insecticides have been identified across the world (Nauen 2007, Coleman et al. 2017), including resistance to insecticide-treated bed nets, a major intervention strategy used in some African countries to reduce malaria transmission (Strode et al. 2014). Thus, insecticide resistance has decreased the efficacy of insecticides to suppress mosquito populations all over the world.

Identifying and monitoring the biochemical or physiological mechanisms associated with resistance is crucial for the development of resistance countermeasures. The three major mechanisms of insecticide resistance include enhanced metabolism (Field et al. 2001), reduced target site sensitivity (Newcomb et al. 1997, ffrench-Constant 1999, Rinkevich et al. 2013) and reduced cuticular penetration (Balabanidou et al. 2018). Enhanced metabolism often results from increased activities of detoxifying enzymes and is widely studied in insecticide resistance pests (Ishaaya 1993, Scott 1999, Hemingway 2000, Li et al. 2007, Pavlidi et al. 2018, Vontas et al. 2018). Esterases are a group of detoxifying enzymes that hydrolyze ester-containing chemicals
into more water-soluble products and have been studied extensively as a major mechanism of insecticide resistance (Raymond et al. 1993, Rider et al. 1998, Jackson et al. 2013a, Wei et al. 2020).

Most insecticides used to manage adult populations of mosquitoes contain esters (e.g., organophosphate and pyrethroid), and enhanced esterase activities are often associated with resistance to these insecticides. As a result, rapid measurement of elevated esterase activities is widely used as an indicator of metabolic resistance. Many such studies use a non-insecticide, model substrate, (α-naphthyl acetate; α-NA) in colorimetric assays to rapidly measure activity (Lewis and Madge 1984, Hemingway 1985, Grant et al. 1989, DeSilva et al. 1997, Baker et al. 1998, Gordon and Ottea 2012). However, insects in the field are exposed to ester-containing insecticides such as malathion, which is hydrolyzed by elevated esterases of resistant insect pests, and the chemical structures of these two substrates (α-NA and malathion) vary considerably. Malathion is an organophosphate insecticide with two ethyl esters which is hydrolyzed by esterases into malathion monoacid and diacid (Matsumura and Hogendijk 1964, Berkman 1994). In contrast, α-NA is an aromatic, methyl ester that is hydrolyzed to α-naphthol.

The relationship between esterases hydrolyzing α-NA and malathion is variable in insecticide-resistant pests. Earlier studies (Oppenoorth and van Asperen 1960, Hemingway 1985, Hughes and Raftos 1985, Ziegler et al. 1987, Baker et al. 1998, Smyth et al. 2000, Gao et al. 2006) have shown that malathion hydrolysis in resistant pests is due to an esterase distinct from those hydrolyzing α-NA. Newcomb et al. (1997), has reported that a mutation in an esterase gene resulting in smaller amino acid residue in malathion-resistant sheep blow fly, *Lucilia cuprina*, altered substrate selectivity between α-NA and malathion. In previous studies (Oppenoorth and van Asperen 1960, Beeman and Schmidt 1982, Hughes and Raftos 1985, Chen
and Sun 1994, Sakata and Miyata 1994, Whyard et al. 1994), a specific esterase, malathion carboxylesterase, has been associated with malathion resistance and in some cases, increased malathion carboxylesterase activity is associated with a decreased α-NA hydrolysis (Oppenoorth and van Asperen 1960, Beeman and Schmidt 1982, Hughes and Raftos 1985). However, additional studies (Hughes and Raftos 1985, Malcolm and Boddington 1989, Ketterman et al. 1992) have shown the variable relationship between the esterases hydrolyzing malathion and α-NA in different insect pests. For example, increased in malathion hydrolysis with no change in α-NA hydrolysis was found in malathion-resistant Anopheles and Culex tarsalis but no malathion carboxylesterase was found in studies with resistant C. quinquefasciatus (Hemingway 1985, Whyard et al. 1995, Karunaratne and Hemingway 2001). The biochemistry of malathion carboxylesterase is not fully resolved and variable among different species of insects. Therefore, in this study esterases associated with malathion resistance were measured and compared to validate use of the model substrate, α-NA, in measuring esterase activity. Esterase activities towards α-NA or malathion as substrates were measured and compared in field-collected adult female C. quinquefasciatus. Results from this study will help to understand the relation between esterases hydrolyzing insecticide and model substrate α-NA and validate the use of α-NA as a substrate for measuring resistance-associated esterase activity.

3.2. Materials and methods

3.2.1. Chemicals

Iodonitrotetrazolium chloride (INT) (≥98% purity), β-nicotinamide adenine dinucleotide sodium salt (≥95%), alcohol dehydrogenase, diaphorase, 1-naphthyl acetate (α-NA;≥98%), Fast Blue B salt (approx. 95 %), Brilliant Blue G-25 (ultra-pure), sodium phosphate monobasic monohydrate (≥ 98%), sodium phosphate dibasic heptahydrate (98%), glycine (>99%), sodium
dodecyl sulfate (SDS) (99%), acetone (99.5%) and phosphoric acid (ACS grade) were purchased from Millipore Sigma (Burlington, MA). Hydrochloric acid (99.7%), phosphoric acid (85%), potassium chloride (ACS grade) and sodium hydroxide (ACS grade) were purchased from Fisher Scientific (Kansas City, MO). Bovine serum albumin (biotechnology grade) and Tris base (99.9%) were purchased from Amresco (Solon, OH). Absolute ethanol (ACS/UPS grade) was purchased from Pharmco-Aaper (Brookfield, CT). Malathion (99.5%) was purchased from Chem Service (West Chester, PA). Yeast powder was purchased from Solgar (Leonia, NJ) and beef liver powder was purchased from Now Foods (Bloomingdale, IL)

3.2.2. Insects

A reference susceptible laboratory strain (Sebring-S) and field-collected populations of *C. quinquefasciatus* were used. Sebring-S, originally collected and maintained by the USDA Agricultural Research Station (Johnsen 2010), was provided by the Medical Entomology lab at LSU and has no known history of exposure to chemicals. Field populations of *C. quinquefasciatus* were collected from residential sites located at East Baton Rouge Parish and Orleans Parish of Louisiana, US, where malathion and bifenthrin had been applied recently by personnel from the East Baton Rouge Mosquito Abatement and Rodent Control (EBMARC), New Orleans Mosquito, Termite and Rodent Control Board (NOMTRCB) and commercial pest applicators (Table 3.1). There was a total of eight residential sites, with seven (Field A-G) located in East Baton Rouge Parish and one located in Orleans Parish (Field-H).

Egg rafts were collected from field sites throughout the summer and fall of 2020 in black plastic containers containing a mixture of distilled water (4 L) and yeast powder (2 g). Field-collected populations of *C. quinquefasciatus* were used in the same generation in which they were collected. Larval and adult *C. quinquefasciatus* were reared at constant photoperiod (14 h
light:10 hr. dark) and humidity (70%). Larvae were fed daily with a mixture of liver powder and yeast (1:1) and adults were provided with 10% sugar solution. Adult females (7-10 days old) were fed defibrinated chicken blood (Rockland Immunochemical®) using a membrane feeding system (Hemotek®, Blackburn, England).

Table 3.1. Field populations of *C. quinquefasciatus* used in the study.

<table>
<thead>
<tr>
<th>Mosquito population</th>
<th>Parishes</th>
<th>GPS Coordinates</th>
<th>Sprayed chemicals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field-A</td>
<td>East Baton Rouge</td>
<td>30° 25'56&quot; N 91° 9'52&quot;W</td>
<td>Bifenthrin</td>
</tr>
<tr>
<td>Field-B</td>
<td>East Baton Rouge</td>
<td>30° 25'54&quot; N 91° 9'52&quot;W</td>
<td>Unsprayed</td>
</tr>
<tr>
<td>Field-C</td>
<td>East Baton Rouge</td>
<td>30° 26'1&quot; N 91° 9'52&quot;W</td>
<td>Malathion</td>
</tr>
<tr>
<td>Field-D</td>
<td>East Baton Rouge</td>
<td>30° 25'52&quot; N 91° 9'53&quot;W</td>
<td>Unsprayed</td>
</tr>
<tr>
<td>Field-E</td>
<td>East Baton Rouge</td>
<td>30° 26'59&quot; N 91° 06'40&quot;W</td>
<td>Malathion</td>
</tr>
<tr>
<td>Field-F</td>
<td>East Baton Rouge</td>
<td>30° 23'03&quot; N 91° 07'09&quot;W</td>
<td>Malathion</td>
</tr>
<tr>
<td>Field-G</td>
<td>East Baton Rouge</td>
<td>30° 23'03&quot; N 91° 9'53&quot;W</td>
<td>Malathion</td>
</tr>
<tr>
<td>Field-H</td>
<td>Orleans</td>
<td>30° 00'58&quot; N 91° 03'05&quot;W</td>
<td>Malathion</td>
</tr>
</tbody>
</table>

3.2.3. Biological assay

Susceptibility of *C. quinquefasciatus* to malathion was examined using a topical bioassay. Adult females (3-7 days old) were anesthetized with CO₂ for less than 1 min then treated with varying concentrations of malathion in 0.5µl acetone on the thoracic dorsum using a syringe fitted with a repeating dispenser (Hamilton, Reno, NV). This experiment was repeated 3 times on 3 different days using different collections of *C. quinquefasciatus*. Control insects were treated with acetone only. Treated females were placed in waxed paper cups (270 ml capacity) fitted with fine mesh on the top. Treated and control mosquitoes were fed via cotton balls soaked in a 10% sucrose solution and held in environmental chambers as described above. Mortality was recorded 18 hours after treatment and was defined by an inability to right themselves after being
flipped onto their dorsum. Control mortality was below 5% in all experiments and was corrected using Abbott’s formula (Abbott 1925). The LD₅₀ values along with significance of slopes were measured following Probit analysis using the ecotox package in R (Team 2013b).

3.2.4. Biochemical assays

For enzyme assays, mosquitoes (adult female; 3-7 days old) were homogenized in 1.15% Kcl using 10 strokes of an all-glass mortar and pestle, then centrifuged at 4°C for 10 mins at 15,000 g. The supernatant from this spin was used as an enzyme source immediately after preparation. Esterase activity towards α-NA was measured using the colorimetric assay described by Gomori (1952) as modified by van Asperen (1962) and Grant et al. (1989) in polystyrene 96-well flat bottom microplates (Costar, Cambridge, MA). A stock solution of α-NA (30 mM in acetone) was diluted 100-fold with buffer (0.1 M sodium phosphate), and reactions were started by the addition of 200 µl of α-NA (0.22 mM final concentration) to 20 µl of either enzyme homogenate (12-15 µg protein) or buffer (control). Reactions were stopped after 10 mins. by addition of 50 µl Fast Blue B dye (2.18 mM final concentration). Optical density of reactions was measured at 570 nm using a Thermomax microplate reader (Molecular Devices, Palo Alto, CA), and converted to µmol/min using an experimentally derived extinction coefficient for α-naphthol (2.6 µ𝑀⁻¹ 270 µl).

Esterase activity towards malathion was measured using the colorimetric assay described by Talcott (1979). The reaction mixture contained 50 µl enzyme homogenate (15-18 µg protein) and 200 µl of an NADH-regenerating system containing (final concentration): NAD (1.6 mM), alcohol dehydrogenase (46.35 Units), INT salt (0.66 mM) and diaphorase (0.186 Units). Reactions were started by addition of 1 µl malathion (300 µM) to the reaction mixture. Reduction of INT was measured as the rate of change in optical density for 20 mins at 500 nm
using a Thermomax microplate reader (Molecular Devices, Palo Alto, CA). First order reaction rates were converted to nmol/min using the experimentally derived extinction coefficient of reduced INT (25.7 μM⁻¹ 250 μl). Protein concentrations were measured by the method of Bradford (1976) using bovine serum albumin as the standard.

Conditions for measurement of esterase activity towards both substrates, (α-NA and malathion) were optimized using homogenates of Sebring-S adults in reactions with varying pH and protein content. The buffers used in optimizations of esterase activity were 0.1M sodium phosphate buffer (pH: 6.2-8.6), 0.1 M Tris-HCl (pH: 7.2-9.5), and 0.1 M Glycine-NaOH (pH:7.8-10.6). Similarly, varying amount of proteins (2.8-22 and 2-55 μg) were used to optimize esterase activities towards α-NA and malathion, respectively.

All statistical analyses were conducted in R (Team 2013b). Data from bioassay from both lab and field strains of C. quinquefasciatus were subjected to probit analysis using the function LC_probit from the package ecotox (Hlina et al. 2019). Linear regression with function lm was used to find the correlation between esterase activities and LD₅₀.

3.3. Results

3.3.1. Malathion resistance in field-collected Culex quinquefasciatus

Resistance to malathion in field-collected mosquitoes was relatively low and ranged from 3.2 (Field-F) to 10.4 (Field-H) -fold (Table 3.2).

Table 3.2. Susceptibility of C. quinquefasciatus towards malathion.

<table>
<thead>
<tr>
<th>Strain</th>
<th>LD₅₀ (95% CI)¹</th>
<th>Slope (SE)</th>
<th>Chi-square</th>
<th>RR²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sebring-S</td>
<td>3.4 (2.8-3.9)ᴬ</td>
<td>3.3 (0.5)</td>
<td>19.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Field-A</td>
<td>15.9 (14.3-17.3)ᴮ</td>
<td>2.8 (0.6)</td>
<td>14.7</td>
<td>4.7</td>
</tr>
</tbody>
</table>

(Table Cont’d)
<table>
<thead>
<tr>
<th>Strain</th>
<th>LD₅₀ (95% CI)¹</th>
<th>Slope (SE)</th>
<th>Chi-square</th>
<th>RR²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field-B</td>
<td>15.6 (10.4-16.6)ᴮ</td>
<td>5.9 (0.8)</td>
<td>9.1</td>
<td>4.6</td>
</tr>
<tr>
<td>Field-C</td>
<td>28.3 (24.7-33.6)ᴱᴱ</td>
<td>2.4 (0.4)</td>
<td>11.0</td>
<td>8.4</td>
</tr>
<tr>
<td>Field-D</td>
<td>21.2 (19.1-23.2)ᴰ</td>
<td>4.8 (0.7)</td>
<td>14.0</td>
<td>6.3</td>
</tr>
<tr>
<td>Field-E</td>
<td>20.5 (17.3-25.1)ᴰᴰ</td>
<td>3 (0.7)</td>
<td>5.8</td>
<td>3.2</td>
</tr>
<tr>
<td>Field-F</td>
<td>10.6 (9.1-12.3)ᴱ</td>
<td>4.0 (0.8)</td>
<td>0.8</td>
<td>3.2</td>
</tr>
<tr>
<td>Field-G</td>
<td>12.1 (10.7-13.8)ᴮ</td>
<td>4.8 (0.8)</td>
<td>1.7</td>
<td>3.6</td>
</tr>
<tr>
<td>Field-H</td>
<td>34.8 (31.2-37.9)ᴱ</td>
<td>4.42 (0.99)</td>
<td>2.8</td>
<td>10.4</td>
</tr>
</tbody>
</table>

¹ ng malathion/insect (95% Confidence Interval). Values followed by the same letter are not significantly different (P>0.05)

² RR=LD₅₀ of field-collected/LD₅₀ of Sebring-S

3.3.2. Optimal conditions for assays

Relationship between pH and esterase activity differed in assays with α-NA and malathion (Figure 3.1). pH Optima for esterase activity with α-NA was broad with peak activity observed at a value of 8.0. However, two different pH optima were observed for esterase activity with malathion: the major optimum at pH 8 and a minor peak at pH 9. In addition, relationships between the amount of protein and esterase activities were linear with both substrates; α-NA and malathion (Figure 3.2). Rate of hydrolysis of both substrates, α-NA, and malathion, was constant at protein concentration ≤20 µg/assay.
Figure 3.1. Optimization of pH for measurement of esterase activity towards α-NA (A) and malathion (B) using buffers containing 0.1 M glycine NaOH (circle), 0.1 M sodium phosphate (triangle), and 0.1 M Tris-HCL (square). Points represent mean activity (± SD) based on 3 determinations with different homogenates prepared on different days.
Figure 3.2. Hydrolysis of α-NA (A) and malathion (B) in reaction containing varying amount of protein. Points represent mean activity (±SD) based on 3 determinations in triplicates.
3.3.3. Esterase activity in field-collected *Culex quinquefasciatus*

Esterase activities toward both α-NA and malathion were associated with malathion resistance in field-collected *C. quinquefasciatus*. (Figure 3.3). Esterase activity towards α-NA was lowest in the susceptible reference lab colony (3.5 μmol/min*mg protein), and was 2.6- to 5.1-fold higher in field-collected strains. Similarly, esterase activities towards malathion were

![Graph](image_url)

**Figure 3.3.** Association of esterase activity with malathion resistance in *Culex quinquefasciatus*. Points represent mean activity (±SD) based on 3 determinations with different homogenates on different days.
lowest in the susceptible reference lab colony and were 1.3- to 2.7-fold higher in field-collected strains. Finally, esterase activities measured in field strains towards α-NA and malathion were highly correlated (R²=0.89; Figure 3.4).

Figure 3.4. Correlation between esterase activity towards α-NA and malathion. Points represent mean activity (±SD) based on 3 determinations with different homogenates on different days.

3.4. Discussion

Early detection of insecticide resistance is essential to develop resistance countermeasures and depends on accurate and rapid biological and biochemical tests to monitor resistance and detect associated mechanisms. Most studies measure esterase activity with α-NA as a rapid biochemical test to monitor resistance. However, in the field, pests are exposed with ester-containing insecticides such as malathion, which is structurally distinct from α-NA. Thus, it
may be imprudent to use conventional non-insecticide substrates to measure esterase activity without knowing the relation between esterase hydrolyzing the model and insecticide substrates.

A positive correlation was found between esterase activities hydrolyzing the \( \alpha \)-NA and malathion. In addition, the pH optima for esterases hydrolyzing \( \alpha \)-NA overlap with the major pH optima for esterase hydrolyzing malathion. The correlation between the activities and overlapping pH optima suggest that esterases hydrolyzing model substrate, \( \alpha \)-NA may be the same esterase hydrolyzing malathion in field-collected \textit{C. quinquefasciatus}. An earlier study from Karunarathne and Hemingway (2001) found no detectable malathion carboxylesterase activity in malathion-resistant \textit{Culex quinquefasciatus} although it was present in the homogenates of \textit{Anopheles culicifacies} and \textit{Anopheles subpictus}. Similarly, a malathion-resistant population of \textit{Culex tarsalis} expressed an esterase that hydrolyzes both \( \alpha \)-NA and malathion with similar enzyme kinetics, in addition to kinetically distinguished esterase that hydrolyzes malathion only with high affinity. Although this study and others (Beeman and Schmidt 1982, Hemingway 1985, White and Bell 1988, Spencer et al. 1998, Newcomb et al. 2005), have reported the presence of malathion-specific esterase, it is unique in that it is found mostly in highly resistant insect pest specifically towards malathion and is located in the mitochondria of high level of malathion-resistant insect pests (Ziegler et al. 1987, Whyard et al. 1995).

In the current study, the level of malathion resistance in the field collected \textit{C. quinquefasciatus} was relatively low and ranged from 3.6- to 10.4-fold. In addition, resistance in the field populations of \textit{C. quinquefasciatus} was higher (84 to 150-fold) to other insecticides such as the pyrethroids lambda cyhalothrin and bifenthrin (data not shown). Most earlier studies have shown elevated malathion carboxylesterase associated with high levels of malathion resistance. For example, higher level of malathion carboxylesterase was observed in population of \textit{Culex}
*tarsalis* that were 150-fold resistance towards malathion. However, no detectable malathion carboxylesterase was found in field population of *C. quinquefasciatus* broadly resistance to other insecticides. In the current study low level of nonspecific malathion resistance and exposure to broad range of xenobiotics might have gained broad general substrate specificity than high specificity towards malathion for esterase. Substrate specificity for enzymes doesn’t simply depend on the relative affinity of the substrates for the enzymes, but also depends on the ratio of Kcat/km values for their reaction (Eisenthal et al. 2007). According to the study (Whyard et al. 1995), kcat/km ratio of malathion carboxylesterase(type II) which is common in cytosol of both susceptible and resistant strains, is low compared to the general esterase that might have made the malathion less favored than α-NA as a substrate of esterase in the field- population as shown in our study.

The pH optima experiment showed two peaks, major and minor at pH 8 and pH 9, respectively in laboratory population of *C. quinquefasciatus* (Sebring-S). To confirm if that small peak at pH9 is associated with specifically hydrolyzing malathion, esterase activity hydrolyzing malathion and α-NA at pH8 and 9 were measured and compared in different field collected strains. No change in esterase activity peaks at pH8 and 9 (higher activity at pH8 than 9; data not shown) suggested that there was no specific esterase hydrolyzing malathion at pH9 in field-collected population of *C. quinquefasciatus*. The presence of small portion of esterase that hydrolyze malathion in lab population was also reported in earlier study (Townsend and Busvine 1969, Wood et al. 1985, Chang and Whalon 1987, Chen and Sun 1994, Chiang and Sun 1996) and has suggested that this esterase is also common in susceptible populations has low turnover and not efficient compared to unique mutant specific esterase (Hemingway 1985, Hemingway and Karunaratne 1998)
Finally, our study demonstrated that esterases activities toward both α-NA and malathion, are correlated and highly associated with malathion resistance in *C. quinquefasciatus*. Esterase has been shown in previous studies to be associated with insecticide resistance in a number of insect pest (Field et al. 1988, Field et al. 1994, FIELD et al. 1999, Hemingway et al. 2000, Gordon and Ottea 2012). Similarly, our study supports the previous studies and suggest that measurement of esterase activity towards α-NA is a suitable biomarker of malathion resistance in *C. quinquefasciatus*. However, in the future, exploring and comparing the changes in esterase activity using both substrates, α-NA and malathion with loss or gain of malathion resistance in *C. quinquefasciatus* would further clarify the role and type of esterases associated with malathion resistance in *C. quinquefasciatus*. 
CHAPTER 4. ASSOCIATION BETWEEN THE EXPRESSION OF CANDIDATE GENES ENCODING DETOXIFYING ENZYMES AND MALATHION RESISTANT CULEX QUINQUEFASCIATUS

4.1. Introduction

Mosquitoes pose a great threat to public health by carrying pathogens that cause high morbidity and mortality in humans. Historically, insecticides have been used as a powerful weapon to fight against these mosquito vectors specifically in zones with high risk of mosquito associated diseases (Enayati and Hemingway 2010, Liu 2015). Insecticides, especially DDT, played a major role in past campaigns to eradicate malaria worldwide (Nájera 1999, Nájera et al. 2011). Currently, the WHO relies on pyrethroid insecticides for aerial application and to treat bed nets to suppress mosquito vectors (Kupferschmidt 2016). However, development of resistance in mosquitoes against these chemicals increasingly challenges efforts to suppress mosquito populations (Hemingway and Ranson 2000, Ranson and Lissenden 2016). Currently, insecticide resistance is widespread: increased incidence and magnitude of resistance in mosquitoes to all major classes of insecticides have been identified across the world (Coleman et al. 2017).

Enhanced metabolism is one of the major mechanisms of insecticide resistance in mosquitoes and other insect pests (Hemingway 2000, Li et al. 2007). The three major groups of metabolic enzymes that have been associated with insecticide resistance include cytochrome P450 monooxygenases (P450s), esterases, and glutathione S-transferases (GSTs) (Plapp 1976, Li et al. 2007), which have been studied extensively as a major mechanism of insecticide resistance in mosquitoes (Raymond et al. 1993, Rider et al. 1998, Jackson et al. 2013a, Wei et al. 2020). These enzymes detoxify insecticides rapidly and efficiently because of selection for qualitative or quantitative changes in expression of resistance-associated genes. Qualitative changes in gene
expression results in enzymes with higher catalytic rate for insecticide substrates, whereas quantitative changes produce greater quantities of enzymes that metabolize or sequester these chemicals.

Esterases exist as families of detoxifying enzymes that hydrolyze ester-containing insecticides into more water-soluble and less toxic products (Aldridge 1973, Oakeshott et al. 2010). Most insecticides used to suppress populations of adult mosquitoes contain esters and increased expression of genes encoding esterase enzymes is often associated with insecticide resistance (Cao et al. 2008, Liu 2015, Shin and Smartt 2016, Vivekanandhan et al. 2021).

Although there are many esterase genes with known and unknown function in the southern house mosquito, *Culex quinquefasciatus* (Yan et al. 2012), *EST-2* and *EST-3* are directly involved in insecticide resistance and are well-studied (Wirth et al. 1990, Ketterman et al. 1993, Vaughan et al. 1997, Talipouo et al. 2021, Vivekanandhan et al. 2021). Increased expression of these genes, either through qualitative or quantitative changes have been reported in insecticide-resistant *C. quinquefasciatus* (Hemingway 2000). Along with *EST-2* and *EST-3*, upregulation of genes encoding P450 and GST enzymes such as *CYP4C52, CYP6AA7, GST-1-1, GST-E2*, and *GST-theta* have been reported frequently in insecticide-resistant *C. quinquefasciatus* (Yang and Liu 2011, Reid et al. 2012, Liu 2015, Li et al. 2016, Kothera et al. 2019, Talipouo et al. 2021, Gong et al. 2022). However, many such studies have not firmly established the association between insecticide resistance with altered expression of these genes.

In the current study we have measured the relative expression of *CYP4C52, CYP6AA7, EST-2, EST-3, GST-1-1, GST-E2*, and *GST-theta* genes in both susceptible laboratory and resistant field strains of *C. quinquefasciatus*. Similarly, we have examined the role of *EST-2* and *EST-3* in the susceptibilities towards malathion in *C. quinquefasciatus* in both susceptible and
resistant strains. Finally, we have determined the association between malathion resistance and expression of EST-2 and EST-3 in field-collected *C. quinquefasciatus*. Results from this study will help to elucidate the role of these genes in malathion-resistant *C. quinquefasciatus*

4.2. Materials and methods

4.2.1. Insects

A reference-susceptible lab strain (Sebring-S) of *C. quinquefasciatus* with no known history of exposure to insecticides was originally obtained from the United States Department of Agriculture Research Station in Gainesville, FL and was provided by the Medical Entomology Lab at LSU. Field populations of *C. quinquefasciatus* were collected from residential sites located in East Baton Rouge and Orleans Parishes (Louisiana, U.S), at which malathion and bifenthrin had been applied recently by personnel from East Baton Rouge Mosquito Abatement and Rodent Control, New Orleans Mosquito, Termite and Rodent Control Board and commercial pest applicators (Table 4.1). There was a total six residential sites, with five (Field A-E) located in East Baton Rouge Parish and one located in Orleans Parish (Field-F). Egg rafts were collected from these field sites using black plastic containers containing yeast powder (2mg) and distilled water (4 L). Field-collected populations of *C. quinquefasciatus* were used in the same generation in which they were collected. Larval and adult *C. quinquefasciatus* were reared at constant temperature (28 °C), photoperiod (14 h light:10 h dark) and humidity (70%). Larvae were fed daily with a mixture of liver powder and yeast (1:1) and adults were provided with 10% sugar solution. Adult females (7-10 days old) were fed on defibrinated chicken blood (Rockland Immunochemical®) using a membrane feeding system (Hemotek®, Blackburn, England).
Table 4.1. Field populations of *C. quinquefasciatus* used in the study.

<table>
<thead>
<tr>
<th>Mosquito population</th>
<th>Parishes</th>
<th>GPS coordinates</th>
<th>Sprayed chemicals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field-A</td>
<td>East Baton Rouge</td>
<td>30°25'56&quot; N 91°9'52&quot;W</td>
<td>Bifenthrin</td>
</tr>
<tr>
<td>Field-B</td>
<td>East Baton Rouge</td>
<td>30°25'54&quot; N 91°9'52&quot;W</td>
<td>Unsprayed</td>
</tr>
<tr>
<td>Field-C</td>
<td>East Baton Rouge</td>
<td>30°26'1&quot; N 91°9'52&quot;W</td>
<td>Malathion</td>
</tr>
<tr>
<td>Field-D</td>
<td>East Baton Rouge</td>
<td>30°25'52&quot; N 91°9'53&quot;W</td>
<td>Unsprayed</td>
</tr>
<tr>
<td>Field-E</td>
<td>East Baton Rouge</td>
<td>30°26'59&quot; N 91°06'40&quot;W</td>
<td>Malathion</td>
</tr>
<tr>
<td>Field-F</td>
<td>Orleans</td>
<td>30°00'58&quot; N 91°03'05&quot;W</td>
<td>Malathion</td>
</tr>
</tbody>
</table>

4.2.2. Biological Assay

Susceptibility of adult *C. quinquefasciatus* to insecticides was examined using a topical bioassay. Adult females (3-7 days old) were anesthetized with CO₂ for less than 1 min then treated with 0.5 µl of acetone containing malathion on the thoracic dorsum using a syringe fitted with a repeating dispenser (Hamilton, Reno, NV). Control insects were treated with acetone only. Treated females were placed in waxed paper cups (270 ml capacity) fitted with fine mesh on the top. Treated and control mosquitoes were fed via cotton balls soaked in a 10% sucrose solution and held in environmental chambers as described above. Mortality was recorded 18 hours after treatment and was defined by an inability of treated insects to right themselves after being flipped onto their dorsa. A full range of doses was used to measure malathion susceptibility in the laboratory-susceptible, Sebring-S, and field-collected populations of *C. quinquefasciatus* and log dose/mortality relationships were estimated following Probit analysis using the ecotox package in R.

4.2.3. RNA extraction and gene expression

Total RNA was extracted from 3-7 days adult females frozen at -80°C using Trizol (Invitrogen) according to manufacture’s instructions. RNA was eluted in 100 µl of nuclease free...
water and stored in -80 °C freezer until needed. The total amount of extracted RNA was evaluated using a nanodrop 1000 spectrophotometer (Wilmington, USA) and qualified by agarose gel electrophoresis.

The relative expression of seven major candidate genes (Table 2) encoding major detoxifying enzymes was examined. Quantitative reverse transcription PCR (qRT-PCR) was performed using SYBR green based Luna ® Universal One Step qRT-PCR Kit from New England Biolab (Ipswich, MA, USA). Each 20 µl reaction contained 11 µl of master mix (1µl of RT enzyme + 10 µl buffer real-time PCR), 1.6 µl of primers (0.1 µM of forward and reverse primers), and 7.4µl of template RNA in nuclease free water. The thermal cycling condition comprised reverse transcription at 65°C for 1 min, 55 °C for 10 mins and 95 °C for 1 min followed by 40 cycles of PCR amplification at 95°C for 15 s and 57 °C for 1 min 30 s. qRT-PCR reactions were read using Quant Studio 6 Flex Real-Time PCR system (Life Technologies). Expression levels of the candidate genes were normalized to the housekeeping gene, Ribosomal protein S7 (RPS7). The Ct value of a particular gene was subtracted from the average Ct value of housekeeping gene (RPS7) to calculate the ∆Ct values. The fold difference was calculated by raising 2 to the ∆Ct powers. The average Ct values RPS7 from different strains of C. quinquefasciatus did not vary by 1 unit. The primers used for each candidate genes are given in the Table 4.2.

Mosquitoes for gene expression studies were insecticide selected within a single generation using diagnostic concentrations of malathion. Relative expression of different candidate genes (i.e., CYP4C52, CYP6AA7, EST-2, EST-3, GST-1-1, GST-E2, and GST-theta) encoding major detoxifying enzymes was measured on Sebring-S individuals that survived following application of LD₅₀ or acetone. In addition, adults from the most resistant population
(Field-F) were used to measure the relative expression of these candidate genes, as well as to examine the expression of EST-2 and EST-3 in individuals that survived application of a diagnostic concentration of malathion. Similarly, six residential sites (Field-A to -F) along with Sebring-S were used to establish the correlation between relative expression of EST-3 and malathion resistance. The role of EST3 on malathion susceptibility in both lab and field strains of C. quinquefasciatus was determined by treating individuals with different discriminating concentrations of malathion in two sets of experiments (for lab and Field-F) in which expression of EST3 was measured in individuals that survived or died following exposure to diagnostic doses. In one set of experiments, adults from Sebring-S were treated with doses corresponding to the LD20 or LD80 of malathion as described above, and expression of EST3 was measured in groups of individuals that died following the application of LD20 (most susceptible) or survived following the application of LD80 (most resistant). Similarly, in a second set of experiments, individuals from Field-F were treated with doses corresponding to LD20 or LD80 of malathion as described above, and expression of EST3 was measured in groups of individuals that died following application of LD20 (most susceptible) or survived following application of LD60 (most resistant).

Table 4.2. Oligo nucleotides for qRT-PCR experiment.

<table>
<thead>
<tr>
<th>Transcript ID</th>
<th>Gene</th>
<th>Primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPIJ006763</td>
<td>RPS7</td>
<td>Forward: TCCGGGCATCTTTGATGTGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: TGCGTGACCTCCACATCACC</td>
</tr>
<tr>
<td>CPIJ018943</td>
<td>CYP4C52</td>
<td>Forward: ATCTGCTCAGCGTGCTGTCG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: TTTCCTCGACCGCGTGGTTAG</td>
</tr>
<tr>
<td>CPIJ005959</td>
<td>CYP6AA7</td>
<td>Forward: GCGGCATGACGGAATATA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: CTGATGTGACCCTCGACGCA</td>
</tr>
<tr>
<td>CPIJ013917</td>
<td>EST-2</td>
<td>Forward: GAGCTCGGGTTAAAGGCACC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: ATCGGCGCCTCCAGAAGATC</td>
</tr>
</tbody>
</table>

(Table Cont’d)
The transcript ID number from the vector base of the *C. quinquefasciatus* genome sequence.

4.2.4. Statistical Analyses

All statistical analyses were conducted in R. Data from bioassays were subjected to Probit analysis using the function `LC_probit` from the package `ecotox` and resulting LD$_{50}$ values were used to measure malathion susceptibility. Pairwise comparisons were made using the Student’s t test and one-way analysis of variance (ANOVA) for multiple sample comparison; a value of $P \leq 0.05$ was considered statistically significant. Linear regression (`lm` function) was used to determine relationship between esterase activities and percent mortality resulting from exposure to diagnostic concentrations.

4.3. Results

4.3.1. Malathion resistance in field-collected *C. quinquefasciatus*

Resistance to malathion in field-collected mosquitoes was relatively low and in the range of 3.2 to 10.4-fold (Table 4.3).

<table>
<thead>
<tr>
<th>Strain</th>
<th>LD$_{50}$ (95% CI)$^1$</th>
<th>Slope (SE)</th>
<th>Chi-square</th>
<th>RR$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sebring-S</td>
<td>3.4 (2.78-3.87)$^A$</td>
<td>3.3 (0.5)</td>
<td>19.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Field-A</td>
<td>15.9 (14.3-17.3)$^B$</td>
<td>2.8 (0.5)</td>
<td>14.7</td>
<td>4.7</td>
</tr>
</tbody>
</table>

(Table Cont’d)
<table>
<thead>
<tr>
<th>Strain</th>
<th>LD50 (95% CI)</th>
<th>Slope (SE)</th>
<th>Chi-square</th>
<th>RR$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field-B</td>
<td>15.6 (10.4-16.6)$^B$</td>
<td>5.9 (0.8)</td>
<td>9.1</td>
<td>4.6</td>
</tr>
<tr>
<td>Field-C</td>
<td>28.3 (24.7-33.6)$^C$</td>
<td>2.4 (0.4)</td>
<td>11.0</td>
<td>8.4</td>
</tr>
<tr>
<td>Field-D</td>
<td>21.2 (19.1-23.2)$^D$</td>
<td>4.8 (0.7)</td>
<td>14.0</td>
<td>6.3</td>
</tr>
<tr>
<td>Field-E</td>
<td>20.5 (17.3-25.1)$^{CD}$</td>
<td>3 (0.7)</td>
<td>0.82</td>
<td>3.2</td>
</tr>
<tr>
<td>Field-F</td>
<td>34.8 (31.2-37.9)$^E$</td>
<td>4.42 (0.99)</td>
<td>2.8</td>
<td>10.4</td>
</tr>
</tbody>
</table>

$^1$ ng malathion/insect (95% Confidence Interval). Values followed by the same letter are not significantly different (P>0.05).

$^2$ RR=LD$_{50}$ of field-collected/LD$_{50}$ of Sebring-S.

4.3.2. Expression profiles of major candidate detoxification genes in laboratory and field strains

The relative expression of seven genes encoding major detoxifying enzymes associated with metabolic resistance was compared between groups of individuals from Sebring-S that survived application of a diagnostic dose of malathion (LD$_{50}$ of malathion). *EST*-3 was most differentially upregulated (18-fold) in these insects (Figure 4.1). Similarly, *CYP4C52* was upregulated 10-fold in survivors of malathion treatment. Finally, *EST*-2, *GST-E2*, and *GST-theta* were downregulated in malathion-treated individuals, but differences were not statistically significant (P>0.05). Detoxification genes *CYP6AA7* and *GST-1-1* were also down regulated 2.5- and 1.7-fold, respectively in malathion-treated compared to acetone-treated individuals of the Sebring-S strain. The relative expression of these six major detoxification genes was also measured in a resistant field strain (Field-F) of *C. quinquefasciatus*. Again, *EST*-3 was most upregulated (4.5-fold) followed by *EST*-2 (2.4-fold) in the resistant field strain compared to the susceptible laboratory strain (Figure 4.2).
Figure 4.1. Expression of detoxification genes in individuals from the Sebring-S strain that survived following application of a LD$_{50}$ of malathion. Bars represent mean relative expression levels of genes (±SD). Asterisks represent statistically significant differences (P<0.05). Inset: Relative expression of EST-3 in enlarged view.

Figure 4.2. Expression of detoxification genes in Sebring-S and Field-F population of $C. quinquefasciatus$. Bars represent mean relative expression levels of genes (±SD). Asterisks represent statistically significant differences (P<0.05). Inset: Relative expression of EST-3 in enlarged view.
In a separate experiment, expression of EST-3 was measured and compared in individuals that had survived or died following application of different diagnostic concentrations of malathion in Sebring-S and Field-F (Figure 4.3). EST3 was highly upregulated in individuals that survived application of the diagnostic concentration of malathion in both lab (Sebring-S) and field strain (Field-F) compared to the dead individuals (Figure 4.3).

Figure 4.3. Expression of EST-3 gene in individuals of the Sebring-S and Field-F populations of C. quinquefasciatus that died or survived following application of discriminating doses of malathion. Expression of EST-3 was measured in individuals that died following application of the LD$_{20}$ of malathion or survived following application of the LD$_{80}$ (Sebring-S) or LD$_{60}$ (Field-F) of malathion. Control insects were treated with acetone only. Bars represent mean relative expression levels of genes (±SD). Asterisks represent statistically significant differences (P<0.05).
*EST-3* was upregulated 2.4-fold in individuals of susceptible laboratory strain that survived following application of the LD$_{80}$ of malathion compared to individuals that died following the application of LD$_{20}$ of malathion. The relative expression of *EST-3* in dead individuals from application of the LD$_{20}$ dose of malathion is similar and not statistically different compared to the acetone treated (control) individuals. Similarly, the relative expression of *EST-3* was highly upregulated in individuals of field strain (Field-F) that survived application of the LD$_{60}$ of malathion compared with individuals that died following the application of the LD$_{20}$ of malathion.

4.3.3. Correlation of *EST-2* and *EST-3* genes expression with malathion susceptibility

Relative expression of *EST-3* and malathion susceptibility in *C. quinquefasciatus* were highly correlated ($R^2 = 0.89$; Figure 4.4). *EST-3* expression was lowest in susceptible laboratory strains and were 3.7 -28.7-fold higher in field-collected strains. However, the association between *EST-2* expression and malathion susceptibility in *C. quinquefasciatus* was not strong ($R^2 = 0.26$).
Figure 4.4. Correlation between expression of genes encoding esterase enzymes \((EST-3\) and \(EST-2\)) and malathion resistance in \textit{Culex quinquefasciatus}.

4.4. Discussion

Enhanced metabolism resulting from upregulation of genes encoding major detoxifying enzymes has been shown previously to be a major mechanism of insecticide resistance. In addition, altered regulation of these genes has been shown in response to environmental stress (Yang et al. 2016, Huang et al. 2017). Therefore, it is crucial to establish the association between the upregulation of these genes and insecticide susceptibility. In the current study, we show that the overexpression of the \(EST3\) gene encoding an esterase is associated with malathion resistance in field strains of \textit{C. quinquefasciatus}.
Results from our study validate use of *EST-3* gene as a biomarker of malathion resistance in *C. quinquefasciatus*. In the current study, a positive correlation was found between malathion resistance and expression of *EST-3* in field-collected *C. quinquefasciatus*. Similarly, high expression of *EST-3* in individuals that survived exposure to diagnostic concentrations of malathion compared to the dead individuals in both lab and field-strain of *C. quinquefasciatus* further verify our finding that *EST-3* plays a role in the malathion resistance observed in these strains. However, the expression of *EST-3* in the individuals treated with acetone (as a control) was similar to that of the dead individuals following the treatment of diagnostic dose of malathion. Control group of individuals in this experiment has possibility of mixing of individual mosquito of different level of malathion susceptibility (from high to low), which might have contributed to low level of expression of *EST-3* compared to the surviving individuals. There might be the possibility of induction of these genes following application of malathion as several studies have shown induction of genes following application of insecticides in different insect pests (Terriere 1983, Scharf et al. 2001, Gong et al. 2013). However, the overexpression of *EST-3* in field collected malathion resistance population of *C. quinquefasciatus* suggests that the expression of *EST-3* and malathion resistance are not associated with induction. The results from this study are similar to earlier studies showing overexpression of *EST-3* in organophosphate- and pyrethroid-resistant *C. quinquefasciatus* (Peiris and Hemingway 1993, Kothera et al. 2019, Talipouo et al. 2021).

Expression of other genes along with *EST-3*, which have been associated with pyrethroid and organophosphate resistance in *C. quinquefasciatus* was measured in both lab and field-collected *C. quinquefasciatus*. In comparison with expression of these genes in Sebring-S individuals, *EST-3* was most highly expressed (18.6-fold) in malathion-treated individuals
followed by \textit{CYP4C52}, which was 12-fold overexpressed. Similarly, in the field-collected population of \textit{C. quinquefasciatus}, \textit{EST-3} was most highly expressed compared to other genes. This again supports the hypothesis that \textit{EST3} is associated with and is a potential biomarker of malathion resistance in \textit{C. quinquefasciatus}. However, there are other genes in laboratory population of \textit{C. quinquefasciatus} that were mostly downregulated in malathion treated individuals for unknown reasons. Several studies examining the expression of genes encoding major detoxifying enzymes have reported the under expression of these genes along with overexpression without clearly elucidating the significance of under-expressed genes (King-Jones et al. 2006, Misra et al. 2011, Epis et al. 2014, Silva Martins et al. 2019). One possible reasons for this may be the fitness cost associated with insecticide resistance, where energy metabolism programs may be altered in response to toxic stress by insecticides (Gao et al. 2022).

In conclusion, expression of \textit{EST-3} and malathion susceptibility is correlated in field-collected strains of \textit{C. quinquefasciatus}, suggesting that \textit{EST-3} is associated with resistance in \textit{C. quinquefasciatus}. To further verify this finding, the effect of knockout of this gene on malathion susceptibility should be examined in the future. Similarly, measuring the expression of this gene in tissues primarily involved in detoxification process such as fat body and Malpighian tubules would more precisely establish the role of \textit{EST-3} in malathion resistance in \textit{C. quinquefasciatus}. 
CHAPTER 5. SUMMARY

Mosquitoes are an important target of pest control as they vector pathogens that are associated with many debilitating human diseases. Mosquitoes have been selected with insecticides for over 100 years, which has dramatically increased the frequencies of insecticide resistance all over the world. Most studies have focused on insecticide resistance in mosquitoes resulting from selection by direct targeted spray of insecticides. However, other aspects of selection are largely undetermined and are important in the development of management strategies for insecticide resistance. For example, non-targeted populations of mosquitoes in agricultural areas are selected with agrochemicals. Similarly, commercial application of insecticide at individual residences impacts the susceptibility of populations of mosquitoes at nearby residences. Finally, selection on one life stage may affect insecticide susceptibility in other life stages. Therefore, in the first objective, I measured and discussed the effects of these non-conventional types of selection by insecticides on susceptibility of *Culex quinquefasciatus*. The susceptibility of chlorantraniliprole, which is applied in rice and sugarcane fields in Louisiana, decreased (5.7 to 12-fold) in populations of *C. quinquefasciatus* collected from near these fields compared to reference field strains. In addition, application of bifenthrin by commercial application at an individual residence increased resistance frequencies to bifenthrin at nearby residential sites. The increased frequencies of resistance, as measured with a diagnostic concentration of bifenthrin, were highly correlated ($R^2=0.92$) between larvae and adult suggesting that selection of adults also confers resistance to larvae. Finally, esterase activities and bifenthrin susceptibility were moderately corelated ($R^2=0.4$ for larvae and 0.52 for adults) suggesting that multiple mechanisms (including metabolism by esterases) were associated with observed resistance. Results from this study suggest that non-conventional selection by
insecticides is a variable to considerate when developing management strategies for populations of *C. quinquefasciatus*.

Accurate and rapid physiological and biochemical assays are essential to elucidate the mechanisms of insecticide resistance, knowledge of which is crucial to develop resistance management strategies. Esterases are one of the major metabolic enzymes associated with resistance to ester-containing insecticides such as malathion, which is sprayed routinely in mosquito abatement efforts. Most studies that have measured esterase activities in the lab use a model substrate, α-naphthyl acetate (α-NA), which is structurally different than the insecticide malathion. Studies have shown that malathion hydrolysis in resistant pests is due to an esterase distinct from that hydrolyzing α-NA. Therefore, it is essential to validate the measurement of esterase activity using model substrates through comparison with esterases hydrolyzing insecticide substrates. This was achieved during the second objective of my study where a tight positive correlation (R²=0.89) between esterase activity hydrolyzing α-NA and malathion was found. In addition, pH optima for esterases hydrolyzing the two substrates were similar. These results validate use of α-NA as a model substrate to measure esterase activity in these strains of *C. quinquefasciatus*. Additionally, malathion resistance in *C. quinquefasciatus* was associated with esterase activity, measured with either α-NA or malathion, suggesting that these esterase activities are suitable biomarkers for malathion resistance in adult *C. quinquefasciatus*.

Upregulation of genes encoding major detoxifying enzymes has been associated with insecticide resistance in many insect pests. Genes encoding esterase enzymes such as *EST2* and *EST3* are overexpressed in many cases of organophosphate resistance in *C. quinquefasciatus*. Many of these studies have focused on characterizing these genes instead of establishing the association between their overexpression and insecticide resistance. The third objective of this
study was to establish the association between expression of a gene encoding an esterase enzyme (EST3) and malathion resistance in *C. quinquefasciatus*. Results suggest that the EST-3 gene might be a potential biomarker of malathion resistance in *C. quinquefasciatus*. A positive correlation was found between malathion resistance and expression of EST-3 in field-collected *C. quinquefasciatus*. Similarly, high expression of EST-3 was found in individuals surviving application of a diagnostic concentration of malathion in both lab and field-strains of *C. quinquefasciatus*. To further validate results, effect of knockout of this gene on malathion susceptibility should be examined in future studies. Similarly, measuring expression of this gene in tissues primarily involved in insecticide detoxification (e.g., fat body and Malpighian tubules) would further establish the role of EST-3 in malathion resistance in *C. quinquefasciatus*. 
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VITA

Vivek Pokhrel was born and raised in Nepal, where he received his bachelor’s degree in agriculture from the Institute of Agriculture and Animal Sciences, Tribhuvan University, Nepal in 2011. After finishing his bachelor’s degree, he was employed as an agricultural instructor for diploma level students in Nepal. Vivek was interested in getting his graduate study from abroad and was accepted as a graduate research assistantship in Dr. Healy’s Lab at Louisiana State University in Fall 2014. He completed his M.S. in Entomology remaining in Dr. Healy’s lab in Fall 2016, where his thesis was to study the effects of truck based ultra-volume mosquito adulticides on honey bees. He later joined Dr. Ottea’s lab at Louisiana State University to purse PhD in Entomology working in insecticide resistance in mosquitoes. Vivek plans to receive his PhD in Entomology in Fall 2022. Upon completion of his doctorate, he plans to work as a postdoctoral researcher in Dr. Franz’s lab at University of Missouri.