Resistance Status of *Aedes albopictus* and *Aedes aegypti* from Alabama and Florida and Analysis Cytochrome P450 Genes Expression Level Compared with Susceptible and Resistant Strains

by

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Abstract

Aedes albopictus and *Aedes aegypti* can transmit severe human diseases including dengue fever and yellow fever. When they are serious, these diseases can lead to human death and economic burden. *Ae*. *albopictus* has already spread from its origin Asia to many continents, including North America. In the United States, this species can be found in Alabama, but the resistance status remains unknown. Part of my experiment analyzed the resistance status. *Ae*. *albopictus* from six locations in Alabama were tested and showed no significant resistance. Two main insecticide classes were used: organophosphates (OPs) and pyrethroids. For OPs, chlorpyrifos, malathion and fenitrothion were used. Malathion had the highest LC50 in these three insecticides ranging from 0.1ppm to 1.2ppm. The resistance status was similar between chlorpyrifos and fenitrothion ranging from 0.003ppm to 0.05ppm and from 0.01ppm to 0.1ppm respectively. For pyrethroids there were five insecticides used: deltamethrin, permethrin, resmethrin, etofenprox, and β-cyfluthrin. Resmethrin had a highest LC50 values ranging from 0.05ppm to 0.4ppm compared with other insecticides followed by permethrin ranging from 0.01ppm to 0.2ppm. In these eight insecticides chlorpyrifos has the highest efficacy while malathion has the last efficacy.

Another part of my experiment was to test the resistance status of field *Ae*. *aegypti* in Florida and make comparison with susceptible (S-Lab) and resistant laboratory strains (PR). The result showed that field strains developed resistance to etofenprox, with a resistance ratio of 1400, permethrin had a ratio of 24, and malathion had a ratio of 11. Field strains developed high tolerance to β-cyfluthrin and chlorpyrifos with resistance ratios reaching 9.7 and 7.6. This strain was still

susceptible to fenitrothion with resistance ratio of 0.3. Based on these results, *Ae*. *aegypti* in Florida has developed resistance and high tolerance to some insecticides.

Cytochrome P450 genes are important in insecticide resistance. In this experiment the genes CYP4H30 and CYP6CB1 were chosen to analyze their expression level. For CYP4H30, there was no difference among AeFl, susceptible S-Lab, and resistant PR strains. But the expression level in AeFl and resistant PR strains were upregulated in CYP6CB1. The expression ratio of AeFl was 5.6 and that of PR strain was 33.3 compared to susceptible S-Lab strain.

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List of Abbreviations

AChE	acetylcholinesterase
CYP	cytochrome P450
DDT	dichloro-diphenyl-trichloroethane
GABA	gamma aminobutyric acid
GST	glutathione S-transferase
AeFl	field strain of *Aedes aegypti* form Florida
LC_{50}	lethal concentration to kill 50% for a test population
P450	cytochrome P450 monooxygenase
qRT-PCR	quantitative real-time polymerase chain reaction
S-Lab	an insecticide susceptible laboratory mosquito strain
PR	an insecticide resistant laboratory mosquito strain
OPs	organophosphates

Chapter 1: Literature review

1.1 General Biology of *Aedes albopictus* and *Aedes aegypti*

Mosquitoes are a type of small fly which belong to Culicidae (Diptera). They are holometabolous insects that have four life stages: eggs, larvae, pupa and adults. Their mouthpart is made for piercing and sucking. Some lay their eggs near water like *Aedes*, or on the water surface like *Culex*. After hatching, the larvae are aquatic and consume organic material. Female adults need blood meals to get enough protein to lay their eggs. Their hosts are numerous vertebrates: birds, reptiles, amphibians and some fish. During feeding, mosquito saliva can transfer into the host and thus yellow fever, dengue fever, and other human diseases can be transmitted.

Ae. *aegypti* has similar ecology. This species also can lay eggs near water and needs blood meal to lay eggs. During biting it can also transmit some human diseases, such as yellow fever, Zika fever and dengue fever.

1.1.1 Morphology and Life Cycle

Aedes albopictus, also known as the Asian tiger mosquito, is an invasive species (Paupy et al., 2009). There are some similar species, but *Ae. albopictus* can be distinguished by its thorax and legs (Huang 1968). In the thorax there is a white longitudinal stripe in the middle and the remainder is black (Huang, 1968). There are white spots present on the legs in all femora (Huang 1968). The fore and mid tibiae have dark anteriors and white posteriors while the hind tibiae are dark. The fore and mid tarsus are black while the hind tarsus is completely white (Huang, 1968).

Ae. albopictus has four life stages: eggs, larvae, pupae, and adult. The first three stages are aquatic (Kosova, 2003). Each stage can last several days depending on the temperature and food source (Kosova, 2003). In frost, it will stop growing and remain stable; this stage is called diapause and it can last several months. *Ae. albopictus* can also enter diapause when they experience drought conditions. They do not start growing again until the environment is suitable. Temperature not only affects the growing stage but also affects the incubation period. Warmer temperatures lead to shorter incubation periods (Halstead, 2008). *Ae. albopictus* abundancy also increases with higher rainfall (Halstead, 2008).

1.1.2 Ecology of *Ae. albopictus* and *Ae. aegypti*

The period of development and body sizes vary according to temperature and food source. Female and male adult *Ae. albopictus* need to feed on sugar water to prolong its lifespan (Peach & Gries, n.d.). But only females need blood meals for protein to lay their eggs, which are usually laid near water (Peach et al., 2019). They have a wide range of hosts like various animals, such as reptiles (George et al., 2014). They usually feed during mid-day and rest in the morning and at night. They prefer to live where human is active outside so they can feed on human multiple times.

Female *Ae. albopictus* can mate within several days after emerging from the pupa and mating typically happens around dusk. Females can live longer than one month in captivity. But its lifespan is much shorter in the field, which can be affected by factors like temperature, humidity and food source.

Ae. aegypti had similar ecology with *Ae. albopictus*. *Ae. aegypti* also has four life stages and female *Ae. aegypti* needs blood meal to lay eggs. *Ae. aegypti* can feed at dusk and dawn, indoors and in shady areas.

1.1.3 Distribution

Ae. albopictus originated from Asia and came to America by human transport; inside of tires and artificial containers (Hanson & Craig, 1995). It was first found in the continental US in Harris County, Texas, but was introduced in Hawaii before that (Moore & Mitchell, 1997). Currently, the species has been spread to 678 counties in 25 states (Moore & Mitchell, 1997). Like America, it has also spread to other places such as China, Europe, and Africa (Wu et al., 2011). Its rapid spread has contributed to human diseases such as dengue fever (Hahn et al., 2016).

Ae. aegypti originally comes from Africa and it was spread to tropical and sub-tropical regions (Kraemer et al., 2015). This species concentrates in northern Brazil and southeast Asia including all India, but it only can be present in Spain and Greece and temperate North America (Kraemer et al., 2015).

1.2 *Ae. albopictus* and *Ae. aegypti* as Vectors

Ae. albopictus can transmit disease to humans, animals, and even to arthropods. This species can transmit yellow fever, dengue fever, Chikungunya fever, and there is evidence showing that it can transmit the Zika virus, though this is primarily transmitted by *Ae. aegypti* (Grard et al., 2014). Severe dengue fever can be fatal. This species is also involved in veterinary medicine because it can transmit heartworm to dogs and cats. Besides disease transmission to human and animals, it also can transmit *Wolbachia* to arthropods.

1.2.1 Disease Transmission

Because adult females need blood meals to lay eggs, they can transmit some diseases and virus. In humans, *Ae. albopictus* can transmit dengue viruses but it can transmit other viruses too, for example the Chikungunya virus (Paupy et al., 2009). Dengue viruses can lead to some dengue fever and dengue hemorrhagic fever (DHF), in which the mortality can reach 44% (Martina et al.,

2009). Symptoms of these diseases are fever, headache and vomiting (Rigau-Pérez et al., 1998). Due to the spread of these vectors, these diseases have an increasing incidence and geographical distribution. Worldwide dengue cases can reach 50 to 100 million cases, and DHF can reach 250 000 to 500 000 cases (Rigau-Pérez et al., 1998). Dengue fever and DHF can be found in Asia, Africa and North America, and over half population who live in these areas are at risk of infection (Gubler & Clark, 1995; Rigau-Pérez et al., 1998). In order to control these diseases, some regions spend millions of dollars to manage these diseases. In Puerto Rico, the cost of an epidemic was estimated to reach from 6 to 16 million US dollars. In Cuba, the cost could be over 100 million US dollar (Moore & Mitchell, 1997). This creates a large economic burden to society.

Ae. albopictus not only transmits human diseases but it can also transmit some diseases to arthropods, for example, the *Wolbachia* bacteria (Dobson et al., 2004). The main transmission method for these bacteria is vertical transmission but there is also some evidence to show that they can transmit horizontally between species (Zug & Hammerstein, 2012). If it is true, they can infect many arthropods worldwide and according to statistical estimates, that can be up to 66% (Zug & Hammerstein, 2012). These bacteria can affect host reproduction to increase their own population (Hedges et al., 2008).

Ae. albopictus can also be vectors to animals like dogs and cats (Gratz, 2004). It has been known to transmit heartworms in dogs in Southeast Asia, south-eastern (Gratz, 2004).

1.2.2 Management of *Ae. albopictus* and *Ae. aegypti*

Insecticides play an important role in pest control due to their high efficiency and low cost. Some insecticides, such as pyrethroids and OPs, are popular in the world. The pyrethroid resmethrin is popular in the management of pests in agriculture and public health due to its low mammal toxicity, high potency and low resistance (Coats et al., 1989). Pyrethroids are a type of

neurotoxin and work by keeping sodium channels open so that action potentials are sustained causing the organism to lose energy and die (Soderlund et al., 2002). Although pyrethroids have low toxicity in mammals, they have high toxicity in aquatic invertebrates and fish. Because of their low solubility it is easy to be absorbed by aquatic creatures (Coats et al., 1989). However, frequent use of pyrethroids has led to the problem of resistance. For example, in West Africa, resistance has already occurred and may lead to cross resistance (Chandre et al., 1999).

Another popular insecticide is OPs such as malathion. In America and it is used to manage many crop pests. It can be applied to wheat and corn for mosquito control (Bonner et al., 2007). Like pyrethroids, OPs are kinds of neurotoxin that work by inhibiting acetylcholinesterase (AChE) (Fryer et al., 2004). AChE inhibition of neuron cells to prevent them from returning to a resting state after activation, exhausting and killing the insect. OPs can negatively affect the human nervous system (Bonner et al., 2007; Chambers & Oppenheimer, 2004). OPs use can also lead to resistance. *Ae. albopictus* in Florida and New Jersey was found to have significant resistance (Marcombe et al., 2014).

Using insecticides is an effective method to control pests but unwanted effects like resistance is common so other methods should be considered. Integrated vector management (IVM) uses insecticide-treated nets (ITN) or indoor residual spraying (IRS) (Beier et al., 2008). These two methods successfully controlled malaria in Africa (Beier et al., 2008). Insect repellents can decrease disease transmission by preventing mosquito bites (Rose, 2001). The BG-Sentinel Trap is a new non-chemical method that can be used to capture mosquitoes (Maciel-de-Freitas et al., 2006).

1.3 Insecticide Resistance

Widespread use of insecticides promotes resistance and compromises their efficacy (Marcombe et al., 2014). This is a serious global problem. From a phenotypic perspective, insects can survive doses that should be lethal (Hemingway et al., 2002). Resistance can occur by mutation and by environmental pressure (Hemingway et al., 2002). When insecticides are applied susceptible insects that survive, those resistant insects go on to produce future resistant generations. After several generations, resistant insects become the majority and full resistance occurs.

Insecticide resistance is an increasing problem and a challenge in mosquito control. In 1955, the World Health Organization (WHO) planned to eradicate malaria by using IRS with DDT. However, this plan ended quickly, partly because DDT resistance occurred in a wide range of mosquito vectors and WHO had to change plans in 1976 (Hemingway & Ranson, 2000). Later, WHO reported that DDT resistance was observed in areas with 256 million inhabitants (Hemingway & Ranson, 2000). DDT was first applied for mosquito management in 1946, and the first cases of resistance were reported one year later (Hemingway & Ranson, 2000). Resistance problems also exist with newer insecticides, such as organophosphate, carbamates and pyrethroids (Hemingway & Ranson, 2000). Resistance can be very high in some countries. In Saudi Arabia, the resistance ratio for DDT, permethrin, and deltamethrin is more than 1,000 (Amin & Hemingway, 1989). In Africa, America and Europe resistance emerged 25 years ago (Weill et al., 2003). In 1992, 56 *anopheline* and 39 *culicine* mosquitoes as well as body lice, fleas, and ticks, all showed resistance (Brogdon & McAllister, 1998). Resistance to the newest insecticides have been demonstrated. The diamondback moth, *Plutella xylostella* (L.), which is a major lepidopteran pest for vegetables, show resistance to *Bacillus thuringiensis* (Tabashnik et al., 1990).

Management of pests will be a challenge and as more pests survive under high concentrations of insecticide. Increasing higher concentration and frequency of use may improve control but may also have negative effects to humans, the environment, and economies. During the process of using insecticides human can be poisoned, which can range from skin, eyes, and even the nervous system. Even more serious: insecticides may cause cancer in humans (Bassil et al., 2007). Most insecticides are delivered by spraying and they easily to drift to other water, air, and soil and can lead to pollution. Insecticides can harm non-target insects like honeybees (Tosi et al., 2018). The impact on public health, crop losses, bird losses, and groundwater pollution can cost one billion US dollars (Pimentel & Burgess, 2014).

In conclusion, proper selection and use of insecticides can help to slow resistance and to protect environment. Development of new insecticides with different model of action is an alternative. It is important to understand mechanism of resistance and to control the selection process.

1.4 Resistance Mechanisms

There are different types of resistance mechanism that includes: increased metabolic detoxification, target insensitivity, physiological modification, and behavioral resistance. Behavioral resistance is defined as taking some action that decrease the exposure to toxic compounds (Sparks et al., 1989). Behavioral resistance has several mechanisms such as activity changes to avoid insecticide exposure, selection of hosts and habitats, and increased repellency (Sparks et al., 1989). Some insects also have physiological resistance mechanisms that can reduce insecticide penetration, increased transport, and increased storage and excretion (Sparks et al., 1989). Increased metabolic detoxification and target insensitivity can be divided by biochemical mechanisms (Brogdon & McAllister, 1998). Increased metabolic detoxification happens when

enzymes such as esterases, oxidases, or glutathione S-transferases (GST) are increased or modified (Brogdon & McAllister, 1998). Target insensitivity happens when sites of action are less effective or ineffective at major targets like acetylcholinesterase (AChE), gamma-aminobutyric acid (GABA) receptors and sodium channels (Hemingway & Ranson, 2000).

1.4.1 Increased Metabolic Detoxification

Most resistance to insecticides is metabolic. Insects with high-level resistance can be unaffected by all available insecticides (Hemingway et al., 2004). Three major enzymes are involved in the resistance mechanism: cytochrome P450 monooxygenases (cytochrome P450), hydrolases (esterases), and GSTs (Liu et al., 2006). These three enzymes are responsible for metabolizing different types of insecticides. GST can break down DDT, hydrolases can metabolize OPs and carbamates, and monooxygenases can break down pyrethroids and OPs (Hemingway & Ranson, 2000). Therefore, these enzymes can prevent insects from being poisoned and promote excretion of insoluble toxins out of their bodies.

1.4.1.1 Cytochrome P450 Monooxygenases

Cytochrome P450 is a superfamily of heme-thiolate proteins and their spectral absorbance peak is at 450 nm (Danielson, 2002). These enzymes are notable because they can catalyze oxidative, peroxidative, and reduction reactions. Their substrates are also diverse including endogenous or xenobiotic (i.e., environmental pollution, agrochemicals, allelochemicals, steroids, prostaglandins and fatty acids) (Danielson, 2002).

Cytochrome P450s were first found in mammal livers but they can be found in all organisms: animals, fungi, protists, archaea, bacteria, and plants (Lamb et al., 2009; Werck-Reichhart & Feyereisen, 2000). Because of their extremely diverse functions they can be found in all tissues with well-developed regulated expression (Werck-Reichhart & Feyereisen, 2000). Their

amino-acid sequences are diverse, which identity sequences can be as low as 16%, but their structure fold is conservative through evolution and the most conservative structures are related to heme binding and common catalytic properties (Werck-Reichhart & Feyereisen, 2000). Cytochrome P450 enzymes are soluble in prokaryotes, but in eukaryotes, they are usually bound to the endoplasmic reticulum or inner mitochondrial membranes (Werck-Reichhart & Feyereisen, 2000).

1.4.1.1.1 Nomenclature and Classification

Cytochrome P450 are divided into four classes based on how they deliver electrons. Class I needs both FAD-containing NAD(P)H-reductase and an iron-sulfur redoxin as electron donors while class II only needs FAD/FMN-containing NADPH-P450 reductase (Werck-Reichhart & Feyereisen, 2000). Class III does not need molecular oxygen, or an external electron source and class IV receives electrons directly from NADH (Werck-Reichhart & Feyereisen, 2000).

Cytochrome P450 enzymes are encoded by cytochrome P450 genes. They are named such because a liver microsomal pigment (P) can have an absorbtion peak at 450 nm when reduced and saturated with carbon monoxide (René Feyereisen, 1999). Although P450s have other names, including cytochrome P450 monooxygenases, mixed function oxidases (MFOs), polysubstrate monooxygenases (PSMOs), microsomal oxidase, and heme thiolate proteins the simple term “P450” is a clear and most current designation (René Feyereisen, 1999). The nomenclature of genes of P450 enzymes, introduced by Nebrt et al. includes four parts, a CYP prefix followed by a number for the family, a letter for the subfamily, and a number for the individual gene. All members in a family need to have more than 40% of the same amino acid sequence level and, in a subfamily, all members need to have at least 55% identical sequences (René Feyereisen, 1999).

There are over 500 CYP sequences in GenBank and over 50 of them are from insects (René Feyereisen, 1999).

1.4.1.1.2 P450 Mechanism

Mammals and insects have some similarities in P450 systems. The general catalytic cycle is as follows: the oxidized form heme protein binds the substrates and then a single electron from a redox partner is given to P450-substrate complex, and P450 binds oxygen (René Feyereisen, 1999). A second single electron precedes a reaction where molecular oxygen is split and inserts an atom of oxygen into the substrate in a radical reaction with the other atom becoming part of a water molecule (René Feyereisen, 1999). The same P450 enzymes can catalyze different types of reactions and substrates since a P450 enzyme can be broad and narrow; even changing an amino acid in P450 can affect substrate specificity (René Feyereisen, 1999).

1.4.1.1.3 Insect P450

Mosquitoes and fruit flies are both from Diptera. When their P450 were first fully sequenced, the diversity of P450 genes were better understood among insects along with an increasing number and diversity of insect genomes available (R. Feyereisen, 2006). According to analysis, there are four major clades of insect P450s in the insect genome: the CYP2, CYP3, CYP4 clade and the mitochondrial P450 clade (R. Feyereisen, 2006). These clades are responsible different catalytic reactions. Mitochondria P450s are only found in animals and are involved in metabolizing steroid or vitamin D as well as various xenobiotics but it cannot catalyze insect ecdysteroids metabolism. Clade 2 has broader substrate functions, so it is mainly involved in essential physiological functions. Clade 3 has is most prevalent in insects and it is related to xenobiotic metabolism as well as insecticide resistance. Clades 3 and 4 are also common in insect

genes and include a broad range of functions, such as catalyzing xenobiotics and odorant or pheromone metabolism (R. Feyereisen, 2006).

P450 enzymes are involved in diverse biochemical reactions. They are involved in synthesizing insect chemicals like juvenile hormones and ecdysteroids, that are related with insect growth, development and reproduction. They also metabolize some foreign chemicals like insecticides (René Feyereisen, 1999, p. 450).

1.4.1.1.3.1 Metabolism of Endogenous Compound

A study by Hammock of P450 in insect endocrine glands showed that juvenile hormone III was synthesized from methyl farnesoate epoxidation catalyzed by a P450-like enzyme in corpora allata homogenates of *Blaberus giganteus*. A later study of this enzyme in *Locusta migratoria* corpora allata proved that this reaction was catalyzed by microsomal P450 (Helvig et al., 2004). P450s are not only involved in biosynthesis, some studies showed that enzymes like CYP6A1 are also involved in various terpenoid metabolism; producing diepoxides from methyl farnesoate (René Feyereisen, 1999).

P450s are also related to ecdysteroid metabolism (René Feyereisen, 1999). In biosynthesis there are three hydroxylations at C-25, which is metabolized by microsomal enzymes, C-22 and C-2 metabolized by mitochondrial enzymes (René Feyereisen, 1999). Ecdysone converts into 20-hydroxyecdysone, which occurs in many peripheral organs and are catalyzed by P450 enzymes (Rees, 2013). This enzyme system appears in some peripheral tissues, such as fat bodies, midgut and Malpighian tubes. According to tissues and species it may be located in mitochondria and/or microsomes (Rees, 2013). It is likely that this enzyme system is encoded by more than one gene (René Feyereisen, 1999).

P450 can catalyze some other endogenous compounds metabolism. For example, the CYP4CI gene is cloned from fat bodies of the cockroach and appear to be involved in lipid metabolism. This gene expression can be induced by starvation or by injection of hypertrehalosemic hormone in decapitated animals (René Feyereisen, 1999). In house flies, P450s can catalyze the formation of (z)-9-tricosene, a major sex pheromone component, and these enzymes can involve several formation steps (Reed et al., 1994, p. 2).

1.4.1.1.3.2 Metabolism of Xenobiotics

In insects P450s are important for detoxification of chemicals such as insecticides and plant toxins. Their overexpression can lead to increased levels of P450 expression and activity (Liu et al., 2015). When P450 expression and activity levels are increased, insecticide resistance occurs. The main enzymes for metabolizing pyrethroid are P450s and there are 111 P450 enzymes in *Anopheles gambiae* (Ranson et al., 2011). Using microarray-based approaches one can find three repeatedly overexpressed candidate P450 genes for pyrethroid resistance: CYP6M2, CYP6P3, and CYP6Z2 (Ranson et al., 2011). All these three genes can bind to substrates but only CYP6M2 and CYP6P3 have the function of metabolism (Ranson et al., 2011). Other enzyme families are not involved in metabolism but may have other secondary functions such as prevention pyrethroid-induced oxidative stress, catalyzing secondary products from P450s, or decreasing total concentration of pyrethroids through binding (Ranson et al., 2011).

Insect resistance to plant chemicals may affect their resistance to insecticides (Després et al., 2007). Some studies showed that P450s are related to the detoxification of plant chemicals in some herbivores, such as parsnip webworm *Depressaria pastinacella*, *Manduca sexta* and several *Helicoverpa* earworm species (Després et al., 2007). In *Helicoverpa zea*, this species can use volatile plant molecules as signals to overproduce P450s to metabolize toxins. Another

example is the cactophilic *Drosophila* species from the Sonoran Desert, they can regulate P450 genes to adapt to more host plants. Mosquito larvae can overproduce P450s to help digest decaying leaves, broadening their habitat range (Després et al., 2007).

1.4.1.2 Glutathione-S-transferase (GST)

Glutathione-S-transferase is one of the most important enzyme families for detoxification (Oakley, 2011). Most GSTs are cytosolic dimeric proteins but there are some microsomal GSTs that exist in insects, plants, and mammals (Ranson & Hemingway, 2005). GST subunits have two domains, each containing G and H binding sites (Ding et al., 2003). The G binding site is highly conservative, and binds tripeptide glutathione, while the H site or substrate binding site is variable and is mainly composed of residuals with C-terminals (Ding et al., 2003). GSTs were first found in rat livers and were among multiple enzymes that exist in rats and other mammals. Some studies started to classify GSTs according by substrate specification, immunological cross-reactivity or by the order of elution from affinity columns (Ranson & Hemingway, 2005). As a general rule GST that have more than 40% amino acid similarity were classified into one family (Ranson & Hemingway, 2005). There are at least six classes of GSTs that are soluble, and one membrane-bound microsomal class (Ranson & Hemingway, 2005). To classify mosquito GST amino acid similarity, polygenetic relationship, chromosomal location and immunological properties need to be considered (Ranson & Hemingway, 2005).

GSTs are involved into many biochemical reactions including detoxification, and excretion of numerous endogenous and exogenous compounds (Liu, 2015). When transcription of GSTs is upregulated, this can lead to increased level of protein production and enzymatic activity, that in turn lead to insecticide resistance and detoxification of plant toxins (Liu, 2015). The glutathione binds with OPs that can lead to detoxification. Although there is no evidence to show GSTs are

directly related with pyrethroid resistance they may play an important role in detoxifying lipid peroxidation products that are induced by pyrethroids (Enayati et al., 2005). The concentration of GST changes during the different life stages of insects. In *Aedes aegypti*, the concentration increases during larval development, peaking at the pupa stage and then declining in adulthood (Enayati et al., 2005).

1.4.1.3 Esterase

It is difficult to classify esterases due to their overlapping substrate specificity but later an esterase classification was introduced by Aldridge (Hemingway & Karunaratne, 1998). According to the classification, there are A and B esterases (Hemingway & Karunaratne, 1998). B-esterases can be inhibited by paraoxon in progress and can be adjusted by temperature, but A-esterases cannot be inhibited (Hemingway & Karunaratne, 1998). There are no common rules for esterase nomenclature like the P450s and GSTs so in different animals there may be different nomenclature rules (Hemingway & Karunaratne, 1998). Mentlein and partners isolated purified carboxylesterases from a rat liver microsomal and used the natural substrates to classify them, however in *Culex* mosquitoes, the rules of nomenclature are different according to their preference for hydrolyzing the synthetic esters α- and β-naphthyl acetate and electrophoretic mobility (Hemingway & Karunaratne, 1998, 1998).

Esterases are non-specific enzymes and resistance has been reported in some mosquito species (Liu, 2015). Carboxylesterases (COEs), which are important in hydrolyzing a wide range of ester-containing xenobiotics, are widely distributed in insects, mammals, plans and microbes (Feng et al., 2018). In insects COEs are of concern because they detoxify insecticides like pyrethroids (Feng et al., 2018).

Quantitative and qualitative changes can contribute to amplifying genes and upregulation of transcription of COEs (Feng et al., 2018). Under organophosphorus and carbamate insecticide selective pressure, many arthropod species such as mosquitoes, cockroaches and aphids, are seen to overproduce non-specific COEs (Hemingway et al., 2004). In some insects, instead of increasing expression they increase enzyme activity. This mechanism can be found in malathion resistance which can have a narrower cross-resistance compared to increasing quantity of esterases (Hemingway et al., 2004). This example can be found in carboxylesterases from *Musca domesticai* (Feng et al., 2018).

1.4.2 Target Insensitivity

Neurotoxins are major synthetic insecticides because of their rapid action that can stop crop damage and disease transmission. They have at least 11 sensitive targets (Casida & Durkin, 2013). Most insecticides are also nerve poisons. DDT was first introduced in the 1940s followed by OPs in the 1950s, methylcarbamates (MCs) in the 1960s, pyrethroids in the 1970s, and neonicotinoids in the 1990s (Casida & Durkin, 2013). Modification of structure and mutation of gene coding target proteins can lead to target insensitivity (Liu, 2015). There are three major targets: sodium channels, acetylcholinesterase (AChE) and γ-aminobutyric acid (GABA) receptors. Insecticides such as DDT and pyrethroids target sodium channels, while OPs and carbamate target AchE, and Cyclodiene and fipronil insecticides bind to γ-aminobutyric acid (GABA) receptors (Liu, 2015).

1.4.2.1 Sodium Channels

The voltage-gated sodium channels work on the action potential by mediating the increase of sodium penetration. This channel is a transmembrane protein complex formed by a water-filled pore through the lipid bilayer to allow certain ions to pass (Zlotkin, 1999). Three stages in sodium channels can be detected. In the resting stage, sodium channels close to keep sodium ions outside

cells and potassium and chloride ions inside. The second stage occurs when sufficient stimulation arrives and the sodium channels open. In the third stage, sodium ions move into the cells, and the cell membrane depolarizes. After depolarization, the sodium channels close and potassium ions continuously flow out, resulting in repolarization, the cell membrane returns to resting potential after extra potassium ions flow outside the cell and sodium channels are ready for the next stimulation (Dong, 2007).

Pyrethroids (from pyrethrum flower), synthetic pyrethroids, and DDT can be classified by mechanism despite their different structures. Each act at sodium channels to keep them open, thereby prolonging depolarization (Casida & Durkin, 2013). Modification of sodium channel including point mutations or substitutions can lead to DDT and pyrethroids resistance by decreasing the binding affinity of insecticides to proteins. Changing sodium channel gating properties is also a mechanism of resistance (Liu, 2015). Insecticides that have the same mode of action make it easier to develop cross-resistance. When DDT loses its ability to control house flies, pyrethroids also lose their effectiveness. This was confirmed by electrophysiological studies on nerve sensitivity (Casida & Durkin, 2013).

1.4.2.2 Acetylcholinesterase (AChE)

AChE is a major enzyme to regulate the level of acetylcholine to stop nerve impulses, and its inhibition can lead to death (Fournier, 2005). There are two types of definitions for AChE, one is physiological and the other is biochemical (Fournier, 2005). The physiological definition states that only one AChE gene is appears in insects. The biochemical definition states that AChE is an enzyme with the ability to catalyze acetylcholine hydrolysis (Fournier, 2005). According to the biochemical definition, most insects are encoded by two AChEs genes. Genes *Ace*1 and *Ace*2 are found in cotton aphids, *Aphis gossypii* (Li & Han, 2002). One is expressed in the central nervous

system to hydrolyze acetylcholine in the synapse and the other genes are not responsible for resistance (Fournier, 2005).

OPs and carbamates can inhibit AChE so that acetylcholine cannot be hydrolyzed, leading to repeated stimulation of neurons and finally death. Mosquitoes AChE1 and AChE2 enzymes, but only AChE1 is related to mosquito resistance to OPs and carbamate. Its resistance is caused by mutations because mutations can decrease substrate recognition and the catalytic rate (Fournier, 2005; Hemingway & Ranson, 2000). Some insects have already undergone mutations and developed resistance. *Myzus persicae* has developed carbamate resistance, *Bemisia tabaci* OP resistance, and some mosquito species like the *Anopheles albimanus*, *Culex vishnui*, and *Culex pipiens* have developed resistance to both OPs and carbamate (Liu, 2015).

1.4.2.3 γ-aminobutyric Acid (GABA) Receptors

GABA receptors are commonly found in vertebrates and invertebrates (Hemingway et al., 2004). They are a heteromultimeric gated chloride-ion channel and are mainly located in the central nervous system and in neuromuscular junctions (Hemingway & Ranson, 2000). They can be targets for cyclodiene insecticides like dieldrin. Dieldrin resistance was discovered in the 1950s; the GABA receptors involved in this resistance was reported in the1990s. Some studies show that insects that have resistance to cyclodiene can also tolerate picrotoxin and phenylpyrazole insecticides (Hemingway & Ranson, 2000).

Resistance can be developed by substitution of amino acids, for example: the A296G substitution in *An. gambiae* and the A296S substitution in *An. arabiensis*, *An. stephensi*, *An. funestus*, and *Ae. aegypti* (Liu, 2015). Mutation can also lead to resistance. *Drosophila* treated with cyclodienes can develop resistance due to alanine mutations to serine and this resistance can

be longlasting, presumably because of the lack of a selective disadvantage in resistant flies (Hemingway et al., 2004).

1.4.3 Physiological Resistance

Metabolic resistance and target insensitivity physiological resistance is also important in insects. This type of resistance includes decreased penetration through the cuticle, increased storage in fat bodies or other organs, and increased excretion of insecticides.

1.4.3.1 Decreased Penetration

Before insecticides, neurotoxins or contact insecticides arrived at target sites via cuticles and the digestive system. Diminished penetration can reduce the insecticide amount that is absorbed, and insects can then metabolize these insecticides quickly. This lack of specificity can cause resistance to most insecticides and can promote biochemical resistance mechanisms. This type of mechanism is already found in some insects. The strains of *Helicoverpa armigera* from China and Pakistan have a 330-fold and 670-fold resistance compared to susceptible strains. When these strains are used to conduct [^{14}C] deltamethrin metabolism research, the penetration was much slower than susceptible strains over 24 hours (Ahmad et al., 2006). A similar phenomenon was also observed in the house fly with a 15-fold cross-resistance to fipronil, and with a slower penetration than susceptible strains (Wen & Scott, 1999).

Increasing penetration is something that needs to be considered during the process of manufacturing insecticides.

1.4.3.2 Increased Storage

Increasing storage is also involved in resistance. If fewer active ingredients can arrive at their target sites, then insects can avoid being poisoned. Increasing storage does not work alone, it

can have effects on some enzymes. Carboxylesterases (E4) are produced by peach-potato aphids (*Myzus persicae*) that have already developed resistance to many insecticides including OPS, carbamate and pyrethroids (Devonshire & Moores, 1982). This E4 enzyme is available in large amount inside the body: around 3% of total protein in resistant aphids. But it is not an efficient enzyme to hydrolyze insecticides, so its effect is not only determined by hydrolysis, but by concentration (Devonshire & Moores, 1982).

1.4.3.3 Increased Excretion

Increasing excretion can decrease concentration of insecticides decrease the need for the insecticide to be metabolized. ^{14}C-radiolabeled malathion was applied to both resistant and susceptible strains of *Rhyzopertha dominica*, and susceptible strains were found to have slightly higher metabolism. In resistant strains around 30% to 50% kept intact malathion found in the filter (Matthews, 1980). Western flower thrips (*Frankliniella occidentalis*) have a similar phenomenon in developing resistance. Resistant strains to diazinon can show increased metabolism and excretion (Zhao et al., 1994).

1.4.4 Behavioral Resistance

In behavioral resistance, some behavioral action is taken to decrease exposure to insecticides. There are two types of behavioral resistance: stimulus-dependent and stimulus-independent (Georghiou, 1972). Stimulus-dependent behavioral resistance increases the ability to detect a toxic substrate, and there is some reaction such as that to an irritant or repellent. Stimulus-independent behavioral resistance refers to natural avoidance of a certain environment or host (Georghiou, 1972).

This mechanism can be found in the cockroach *Blattella gennanica* (Ross, 1997). Researcher exposed two strains to low-level chlorpyrifos-treated bait and normal food. Survivors

were mated after several generations, increased behavioral resistance was observed in one strain but decreased in the other strain (Ross, 1997). The *An*. *pseudopunctipennis* mosquito is also better at avoiding lethal DDT after 11 years of exposure (Georghiou, 1972).

1.5 Reference

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Chapter 2: Research Goals and Specific Objectives

2.1 Introduction

Aedes albopictus, also known as the Asian tiger mosquito, is an invasive mosquito species that has spread to 28 countries in Europe, North and South America, and to many areas in the Pacific and Indian oceans. It spread from temperate and tropical forest of its native Asia (Benedict et al., 2007; Paupy et al., 2009). Developmental and reproductive data were analyzed under controlled lab conditions and this analysis will be useful to rear this species for further study. The developmental time of larvae from hatching to pupation is related to temperature, the lower the temperature the longer the developmental time. Development can last 7 days at 32˚C to 28 days at 12˚C (Briegel & Timmermann, 2001). Body size was also measured by wing length, which could vary from 10 mm^3 to 57 mm^3 for females, and from 10 mm^3 to 30mm^3 for males (Briegel & Timmermann, 2001). In August 1985 a population of *Ae. albopictus* was found in Harris County in Texas (Rai, 1991). The speed of spread of this species was dramatic. By the summer of 1989, mosquitoes were widely distributed in 18 states in America, including locations like Brownsville, Texas, and Polk County, Florida. They spread throughout the Midwest from Kansas City, Missouri, and Chicago, Illinois, and east to Baltimore, Maryland, in the east (Rai, 1991). Human activities are the major method that promote its spread. Commercial movements of scrap tires are a prime example (Moore & Mitchell, 1997). *Ae. albopictus* can lead to serious health problems because it is able to transmit human diseases and it was first discovered to be involved in a dengue epidemic in 1986 in Brazil (Rai, 1991). *Ae. albopictus* can transmit dengue and dengue hemorrhagic fevers (DHF) but later studies show that this species has the potential to transmit

other diseases, such as eastern equine encephalitis (EEE) and Japanese encephalitis. Dengue and Japanese encephalitis have already been isolated from *Ae. albopictus* collected in the field in America (Moore & Mitchell, 1997). In southeast Asia deaths caused by dengue increased 300-fold on the last 20 years and in America, the total number of dengue cases could reach 3,141,850 (Bonner et al., 2007; Rai, 1991). *Ae. albopictus* was found in Alabama where studies are not completed (JH et al., 1991; Moore & Mitchell, 1997).

Ae. albopictus is a similar species to *Ae. aegypti*, which is also known as the yellow fever mosquito. These two species can coexist. *Ae. aegypti* originated in Africa and has now spread through the tropics by trading and transport ships (Mousson et al., 2005). This species can transmit human diseases, such as dengue fever and yellow fever, and is also a vector for the chikungunya virus (Eisen & Moore, 2013). *Ae. aegypti* prefers to live in human habitats and can enter buildings to feed and rest (Jansen & Beebe, 2009). Females prefer to feed on humans although they can feed on other vertebrates. They only feed a small amount and they are day-biting mosquitoes who can feed on many hosts during its life cycle (Jansen & Beebe, 2009). Dengue outbreaks were recorded in the 19th century and many countries in the Caribbean, North, Central, and South America were involved. This disease has continued to spread (San Martín et al., 2012). The plan for eradication of *Ae. aegypti* was implemented from 1947 to 1970, but this species reinfested from 1971 to 1999 (San Martín et al., 2012).

2.2 Research Goal and Specific Objectives

Ae. albopictus and *Ae. aegypti* can lead to serious health problems. The study of *Ae. albopictus* in Alabama remains incomplete. My long-term goal is to quantify the resistance level in Alabama and describe the molecular resistance mechanism of P450. In order to achieve this goal, the following three objectives should be completed: 1) to test *Ae. albopictus* collected from

different field locations in Alabama with eight different insecticides to obtain an LC_{50}; 2) to test two strains of *Ae. aegypti* one of which is susceptible and the other highly resistant to some insecticides to obtain an LC_{50}; and 3) to use real-time PCR to test the expression level of P450 in susceptible and highly resistant strains.

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Chapter 3: Resistance Status of *Aedes albopictus* from Alabama

3.1 Abstract

The susceptibility of field collected *Aedes albopictus* to malathion, chlorpyrifos, fenitrothion, β-cyfluthrin, deltamethrin, etofenprox, permethrin and resmethrin was tested. *Ae. albopictus* was primarily collected from Birmingham, Dothan, Mobile, Montgomery, Tuscaloosa and Tuskegee Alabama in 2018 and 2019. Results show malathion was the last toxic insecticide in all locations with an LC_{50} ranging from 0.1ppm to 1.2ppm, followed by resmethrin with an LC_{50} ranging from 0.005ppm to 0.4ppm. Chlorpyrifos was the most toxic insecticide in all locations with an LC_{50} ranging from 0.003ppm to 0.05ppm. For resistance response, *Ae. albopictus* response to β-cyfluthrin was heterogenous with a slope ranging 1.8ppm to 3.0ppm compared to other insecticides. *Ae. albopictus* response to etofenprox had a wide-ranging slope of 2.7ppm to 7.1ppm. This result was similar with a study in 2004. This might indicate that resistance development was slow in Alabama, but *Ae. albopictus* had a higher LC_{50} in malathion than other insecticides. In order to succeed in controlling this species, wise use of insecticides could help to control it with fewer negative effects.

Key words: *Aedes albopictus*, insecticide resistance

3.2 Introduction

The Asian tiger mosquito, *Ae. albopictus*, is a day-biting mosquito species and it can transmit dengue virus, which is a human disease (Vezzani & Carbajo, 2008). It originated in Asia, but it now has spread to many continents such as Africa, the Middle East, Europe, North America,

and South America (Gratz, 2004). The introduction of this species in some countries is thought to be a serious problem because it can transmit diseases to humans and animals (Gratz, 2004). In 1979, *Ae. albopictus* was widely found in Albania and it was the first record an *Ae. albopictus* infection outside the Orient and Australasian regions. Later infection was seen in America (Gratz, 2004). At the close of 1995, *Ae. albopictus* was found in 10 Italian locations and 19 provinces. Other countries also reported the infections from *Ae. albopictus* (e.g., Spain, Portugal, Greece, Turkey and France) (Knudsen et al., 1996).

A large population of *Ae. albopictus* was found throughout Harris County, Texas in August 1985. This species is colder-tolerant than *Ae. aegypti* so *Ae. albopictus* may establish in some northern states where *Ae. aegypti* cannot survive (Rai, 1991). *Ae. albopictus* can infect many countries due to its strong environmental adaption. Although it originated from Asian forests it can survive in human environment and even some high-density locations such as Rome, Italy (Paupy et al., 2009). Their larvae have various habitats from natural sites like tree holes and bamboo stumps, to artificial containers such as water containers and used tires (Paupy et al., 2009). Females prefer to feed during the daytime especially in the early morning and late afternoon, and they prefer to feed on humans, but they have a very wide host ranging feeding on such vertebrates from cold- to warm-blooded animals, reptiles, birds and amphibians (Paupy et al., 2009). Due to its strong ecological adaption, it is easy for them to establish in a new location and they can compete with the local species.

3.3 Material and Methods

3.3.1 Mosquito Sample Collection

All *Ae. albopictus* strains were collected in Mobile, Montgomery, Tuscaloosa, Tuskegee, Birmingham and Dothan Alabama, twice every month from 2018 to 2019. Eight traps in each city

were used to collect mosquito eggs. In all traps, a fusion was made by mixing water, glass, and yeast. Paper were placed inside to let mosquitoes lay eggs. Egg collection was done every two weeks and new papers and fusion were placed to trap more eggs.

3.3.2 Mosquito Sample Differentiation

Ae. albopictus can lay eggs on paper but there are some other *Aedes* mosquitoes, such as *Ae. aegypti*, *Ae. japonicus* and *Ae. triseriatus*, can also do that. After sample collection papers with eggs were put into containers to hatch to become adults, these were distinguished by morphology, only *Ae. albopictus* were kept. There were two different characters to distinguish *Ae. albopictus* from other *Aedes* species. *Ae. triseriatus* had hindlegs that are completely black. The other three species have white scale at each hindleg part but only *Ae. japonicus* has an entirely dark 5th hindertasomeres. *Ae. aegypti* looks very similar to *Ae. albopictus* but they can be distinguished by the scutum. For *Ae. aegypti* the scutum has lyre-shaped markings while *Ae. albopictus* only has a single narrow white stripe.

3.3.3 Insecticides

Eight insecticides were chosen to do bioassays and they had different modes of action. Chlorpyrifos (99.3%), Fenitrothion (95.8%), Etofenprox (99.1%), and β-cyfluthrin (99.9%) were purchased by Sigma-Aldrich®; Malathion (99.2%) and Resmethrin (98%) were bought from Chem Service, Inc.; Deltamethrin (99.9%) and Permethrin (94.34%) were provided by FMC Crop (Princeton NJ).

3.3.4 Bioassay Methods

After differentiating, field mosquitoes were fed blood meals to obtain the next generation that was used to do bioassay. Mosquitoes were incubated in 25 C ±2°C under a photoperiod of 12:12h (L:D). (H. Liu et al., 2004).

Acetone was used to dilute insecticides to prepare a stock solution. Different experimental concentrations corresponded to different stock solutions. Different concentration gradients were set up in order to get 0% to 100% mortality. Ten 4th instar larvae with similar shapes were chosen to be put into 20 mL bottles (20 mL disposable scintillation vials VWR®) with 5 mL of tap water. All tests were incubated under 25 ± 2°C and results were checked after 24 hours. All tests were repeated at least three times on different days. Polo-PC software was used to analyze bioassay data and to construct the Probit analysis; mortality was controlled according to Abbott's correction (Liu et al., 2004). Statistical analysis of LC_{50} and LC_{90} values was based on a non-overlap of 95% confidence intervals (CI).

3.4 Result

Ae. albopictus from Tuskegee had the highest tolerance to chlorpyrifos and the LC_{50} value was 0.05ppm among all strains. *Ae. albopictus* from Dothan and Montgomery had the same LC_{50} values as deltamethrin, which was 0.06, but they had different 95% CIs. *Ae. albopictus* from Dothan had a wider 95% CI compared to *Ae. albopictus* from Montgomery. This may indicate *Ae. albopictus* from Dothan had a higher tolerance to deltamethrin. *Ae. albopictus* from Tuscaloosa had the highest tolerance to etofenprox and the LC_{50} was 0.2ppm followed by *Ae. albopictus* from Tuskegee with an LC_{50} reaching 0.18ppm. *Ae. albopictus* from Tuscaloosa also had the highest tolerance to fenitrothion with an LC_{50} reaching 0.1ppm. *Ae. albopictus* from Dothan had the highest tolerance to malathion in all strains and in all insecticides with LC_{50} values reaching as high as 1.2ppm. For permethrin *Ae. albopictus* from Montgomery had the highest LC_{50} reaching 0.2ppm and for resmethrin *Ae. albopictus* from Tuskegee had the highest LC_{50} which had a value of 0.4ppm. For β-cyfluthrin all LC_{50} was lower than 0.1ppm and the highest LC_{50} was from *Ae. albopictus* in Tuskegee reaching 0.08ppm.

Resistance response was also analyzed. For chlorpyrifos strains from Tuscaloosa had the highest slope reaching 6.5 and this was an indicator that the Tuscaloosa mosquito stain was homozygous compared with the Tuskegee strain. As for deltamethrin, although the strains from Dothan and Montgomery had the same LC_{50}, the Montgomery strain had a higher slope than Dothan. This might indicate that the Montgomery strain was homozygous compared to the Dothan strain. The strain from Tuscaloosa had the highest LC_{50} in both etofenprox and fenitrothion but the slope was double for etofenprox compared to fenitrothion. Thus, the Tuscaloosa strain was heterogenous in fenitrothion. The strain from Tuskegee had a similar slope to resmethrin and β-cyfluthrin so they had a similar response to these two insecticides, but they had a different LC_{50} value.

3.5 Discussion

Ae. albopictus from Mobile was tested for LC_{50} and LC_{90} in 2004 and these values were higher compared to this current study (H. Liu et al., 2004). The slopes had a similar result (H. Liu et al., 2004). Because resistance can only be considered when a resistance ratio is 10 in this study *Ae. albopictus* from Alabama can only be considered to have tolerance to these insecticides (Valles et al., 1997). This result may indicate that these strains developed resistance very slowly or they were not exposed to insecticides. The second answer is more likely because there was study that showed that *Ae. albopictus* from Alabama has not been exposed to insecticide for a long time (H. Liu et al., 2004). Organophosphate insecticides such as malathion, chlorpyrifos and fenitrothion were also tested in this study. These three insecticides these strains had highest LC_{50} and LC_{90} in malathion because other organophosphate insecticides were used to control important mosquito species including *Culex nigripalpus*, *Aedes vexans* and *Ochlerotatus taeniorhynchus* in Alabama (H. Liu et al., 2004). But pyrethroids insecticides are currently the most popular insecticides used

to control mosquitoes. Pyrethroid resistance has already been found in some places (N. Liu et al., 2006). Permethrin resistance has already been found in 1993 in Cotonou (Benin) and Kou valley (Burkina Faso) (Chandre et al., 1999). High resistance to pyrethroids and DDT was detected in Guerrero and multiple resistance mechanisms were shown in *Ae. aegypti* from Guerrero, Mexico (Aponte et al., 2013). Resistance was also detected in America where there was significant resistance to DDT and malathion in two Florida populations and in one New Jersey population (Marcombe et al., 2014).

Insecticide resistance can reduce control efficiency and lead to some negative effects such as inhibition of plant growth, and honeybee and aquatic toxicity. Wise use of insecticides and alternative chemicals are important in controlling mosquitoes (Iwasa et al., 2004; Lichtenstein et al., 1962; Weston et al., 2005). Other chemicals such as insect growth regulators (IGR), naturalytes, biolarvicides and bacteria are also used to control pests (Marcombe et al., 2014). *Bacillus thuringiensis israelensis* (Bti) can be used as biological control agents and it has been used in German since 1981 (Becker, 1997). Bti was widely used because of its environmental safety, simple production and high efficiency to specific mosquito genus such as *Aedes* and *Culex*. (Becker, 1997). Bti can persist in the environment for a long time so they can place continuous selection pressure on mosquitoes (Paris et al., 2011). There was study that showed that individual *Ae. albopictus* could develop resistance to Bti only after several generations (Paris et al., 2011). Insect growth regulators (IGRs) are a new approach to control insect pests and they can affect the development of normal insect growth or their progeny (Tunaz & Uygun, 2004). They work mainly on embryotic, larval and nymphal development by affecting metamorphosis and reproduction, which might limit its use (Graf, 1993). IGRs need more time to work compared to conventional insecticides and sometimes they need to be combined with adulticides to have a quick knock-down

effect (Graf, 1993). IGRs can mainly be classified in three categories: (1) juvenile hormone analogues or mimics (2) chitin synthesis inhibitors and (3) others (Graf, 1993). Some studies that tested *Aedes* and *Culex* showed that embryonated eggs are more tolerant than freshly laid eggs and IGRs can affect eggshell morphology leading to abnormal hatching patterns. Diflubenzuron is an IGR example (Suman et al., 2013). Besides applying IGRs for mosquito eggs, they also can be applied to larvae. A study tested 4th instar *Anopheles quadrimaculatus* showed that IGRs had high efficiency to this strain (Dame et al., 1976). Naturalytes are also a kind of biopesticide and are a promising alternative to control storage pests because of their safety to the environment and to humans (Sanon et al., 2010). A study used three laboratory mosquito strains which were susceptible to all insecticides and field collected strains which were resistant to pyrethroid to test spinosad --a type of naturalyte (Darriet et al., 2005). The result showed that there was no difference between susceptible and resistant strains (Darriet et al., 2005). This indicates that spinosad can be an alternative insecticide and some studies indicate that essential oils extracted from plants could also actively control larval *Ae. albopictus* (Dias & Moraes, 2014).

Understanding the resistance mechanism is also important in *Ae. albopictus* management. There are four types of resistance mechanisms: increased metabolic mechanism, target insensitivity, physiological modification, and behavioral resistance. Increased metabolism and target insensitivity are the most common resistance mechanisms but according to several authors the former is the most common (Scott, 1999). Several enzymes are involved in metabolic detoxification such as esterases, oxidases, and GST. Among these enzymes, cytochrome P450-denpendent monooxygenases are an extremely important for the metabolism of xenobiotics and endogenous compounds. P450 enzymes are the most common type in metabolic resistance (Scott, 1999). This kind of resistance mechanism was not only found in mosquitoes but also some other

insects such as the house fly, *Drosophila*, and German cockroach (Scott, 1999). The other important mechanism for resistance is target insensitivity that includes sodium channel, AChE and GABA receptors (N. Liu, 2015). Pyrethroid insecticides work at the sodium channel and mutations of sodium channels can lead to pyrethroid resistance, this is called knockdown resistance (Oliveira et al., 2013). AChE is the target of DDT and carbamate and these insecticides prevent the hydrolysis of acetylcholine leading to a continuous action potential that causes insect death (Hemingway, 2000). Insects develop resistance to these two insecticides by changing the structure of AChE to decrease its sensitivity (Hemingway et al., 2002). The remaining two non-biochemical resistance mechanisms are also important in the development resistance. Physiological modification includes decreased penetration, increased storage, and increased excretion. The house fly can decrease penetration to develop resistance and the Colorado potato beetle can develop permethrin resistance through rapid excretion (Argentine et al., 1995; Plapp & Hoyer, 1968). Behavioral resistance such as decreased or increased movement and increased irritancy or repellency can help insects avoid insecticides (Graf, 1993). Mosquitoes can reproduce very quickly so it is easy to pass down resistance. Mosquito management should not only use one method, it should consider other methods and should use insecticides wisely to help delay the development of resistance.

Table 1. Toxicity of different insecticides to mosquito strains of *Ae. albopictus*

Insecticide	Locations	DF	n	χ^2	LC_{50}[b](CI)	LC_{90}[b](CI)	Slope (SE)
Chlorpyrifos	Birmingham	3	237	0.2	0.003(0.003-0.004)	0.007(0.006-0.01)	3.3(0.4)
	Dothan	3	497	5.2	0.01(0.009-0.02)	0.03(0.02-0.05)	3.3(0.3)
	Mobile	3	262	0.6	0.008(0.006-0.009)	0.02(0.02-0.03)	3.0(0.3)
	Montgomery	3	342	0.3	0.006(0.005-0.006)	0.01(0.01-0.02)	3.8(0.3)
	Tuscaloosa	4	173	0.1	0.011(0.010-0.012)	0.018(0.016-0.023)	6.5(1.1)
	Tuskegee	2	188	2.2	0.05(0.03-0.09)	0.2(0.09-0.6)	2.7(0.3)
Deltamethrin	Birmingham	4	248	1.4	0.01(0.01-0.02)	0.05(0.04-0.08)	2.3(0.2)
	Dothan	3	278	7.7	0.06(0.03-0.1)	0.2(0.1-1.5)	2.2(0.2)
	Mobile	4	307	2.3	0.02(0.02-0.03)	0.1(0.07-0.1)	2.0(0.2)
	Montgomery	3	122	2.2	0.06(0.04-0.08)	0.2(0.1-0.3)	2.8(0.4)
	Tuscaloosa	4	167	1.9	0.020(0.016-0.023)	0.04(0.03-0.05)	4.4(0.6)
	Tuskegee	3	276	6.2	0.02(0.01-0.03)	0.07(0.04-0.2)	2.2(0.2)
Etofenprox	Birmingham	4	307	0.2	0.06(0.05-0.08)	0.2(0.1-0.2)	3.4(0.4)
	Dothan	3	164	3.5	0.07(0.05-0.1)	0.2(0.1-0.6)	2.7(0.3)
	Mobile	3	193	1.8	0.06(0.05-0.07)	0.2(0.1-0.3)	2.7(0.3)
	Montgomery	3	232	0.006	0.06(0.05-0.07)	0.1(0.09-0.1)	5.4(0.7)
	Tuscaloosa	4	229	5	0.2(0.1-0.2)	0.3(0.2-0.4)	7.1(0.9)
	Tuskegee	2	216	1.6	0.18(0.15-0.20)	0.5(0.4-0.7)	3.0(0.3)
Fenitrothion	Birmingham	2	390	2.9	0.01(0.009-0.02)	0.03(0.02-0.08)	3.4(0.3)
	Dothan	3	184	3.4	0.02(0.01-0.03)	0.04(0.03-0.08)	4.4(0.6)
	Mobile	2	293	0.04	0.02(0.01-0.02)	0.03(0.03-0.04)	4.6(0.5)
	Montgomery	3	168	4.7	0.02(0.01-0.02)	0.03(0.02-0.04)	7.8(1.1)
	Tuscaloosa	2	274	2.5	0.1(0.009-0.02)	0.03(0.02-0.1)	3.3(0.3)
	Tuskegee	3	106	3.0	0.03(0.02-0.04)	0.06(0.05-0.1)	4.3(0.7)
Malathion	Birmingham	4	135	1.7	0.8(0.6-1.1)	2.9(2.0-5.1)	2.4(0.3)
	Dothan	3	184	2.2	1.2(1.0-1.5)	3.6(2.6-5.6)	2.6(0.3)
	Mobile	6	249	9.1	0.1(0.06-0.2)	0.5(0.3-1.2)	1.8(0.2)
	Montgomery	2	106	1.4	0.9(0.7-1.2)	1.9(1.5-3.2)	4.2(0.8)
	Tuscaloosa	5	238	5.1	0.3(0.3-0.4)	0.8(0.5-1.3)	3.5(0.5)
	Tuskegee	4	124	4.8	0.6(0.4-0.9)	1.4(1.0-3.6)	3.4(0.6)
Permethrin	Birmingham	4	235	3.4	0.01(0.01-0.02)	0.04(0.03-0.06)	2.7(0.3)
	Dothan	5	144	2.1	0.08(0.07-0.1)	0.1(0.1-0.2)	4.8(1.0)
	Mobile	4	273	0.2	0.03(0.02-0.03)	0.05(0.04-0.06)	4.7(0.7)
	Montgomery	4	178	1.0	0.2(0.1-0.2)	0.5(0.3-0.7)	2.8(0.4)
	Tuscaloosa	3	337	3.0	0.04(0.04-0.05)	0.08(0.07-0.1)	4.7(0.5)
	Tuskegee	3	127	3.4	0.06(0.04-0.08)	0.09(0.07-0.2)	6.1(1.1)
Resmethrin	Birmingham	2	249	3.2	0.05(0.03-0.1)	0.1(0.08-0.7)	3.1(0.3)
	Dothan	3	162	0.02	0.3(0.2-0.4)	0.7(0.5-1.0)	3.5(0.5)
	Montgomery	3	230	0.4	0.2(0.2-0.3)	0.6(0.5-0.9)	2.9(0.3)
	Tuscaloosa	4	246	7.8	0.2(0.1-0.2)	0.3(0.3-0.7)	4.2(0.5)
	Tuskegee	4	151	3.7	0.4(0.3-0.6)	1.4(1.0-2.5)	2.5(0.4)
	Mobile	3	186	5.4	0.09(0.04-0.1)	0.2(0.1-1.0)	3.4(0.5)
β-cyfluthrin	Birmingham	4	248	1.2	0.03(0.02-0.03)	0.1(0.07-0.2)	2.1(0.2)
	Dothan	4	240	1.1	0.06(0.04-0.08)	0.3(0.2-0.5)	1.8(0.2)
	Mobile	4	159	1.1	0.02(0.02-0.03)	0.08(0.06-0.1)	2.4(0.4)
	Montgomery	4	129	0.8	0.06(0.05-0.08)	0.2(0.1-0.4)	2.5(0.4)
	Tuscaloosa	5	393	7.8	0.01(0.01-0.02)	0.04(0.03-0.06)	3.0(0.2)
	Tuskegee	5	179	8.1	0.08(0.05-0.1)	0.3(0.2-0.8)	2.6(0.4)

Figure 1. 50% mortality of *Ae*. *albopictus* from Birmingham, Dothan, Mobile, Montgomery, Tuscaloosa and Tuskegee, the error bars mean 95% confidence intervals.

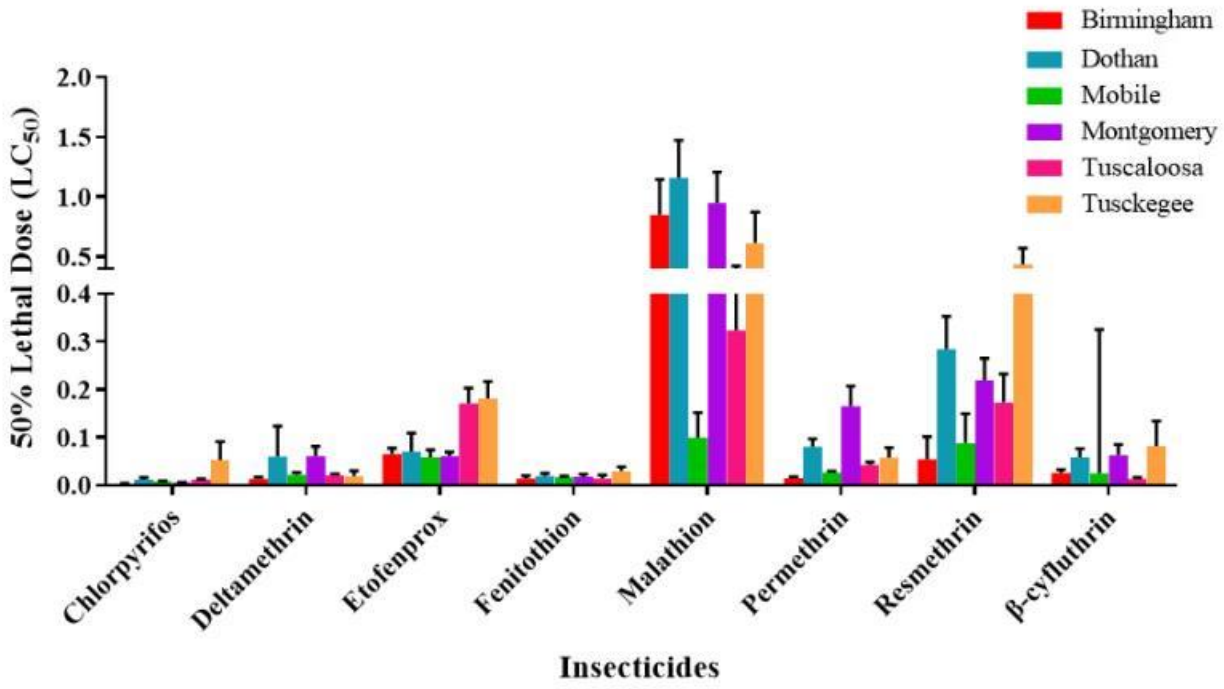
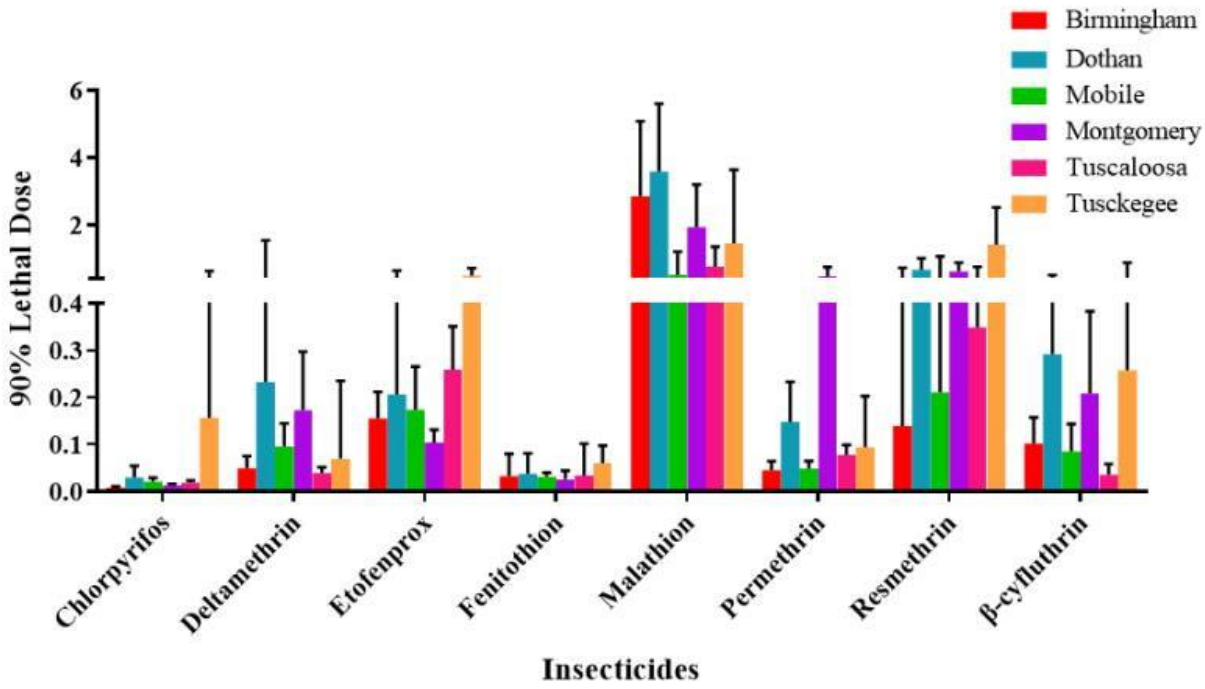


Figure 2. 90% mortality of *Ae*. *albopictus* from Birmingham, Dothan, Mobile, Montgomery, Tuscaloosa and Tuskegee, the error bars mean 95% confidence intervals.



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Chapter 4 Resistance Status of *Aedes aegypti* from Florida Compared with Susceptible Lab and Resistant Strains

4.1 Abstract

Resistance was determined for *Aedes aegypti* from Florida and two laboratory strains that were susceptible (S-Lab) and resistant (PR). The Florida strain showed the highest resistance to etofenprox with resistance ratio of 1400 followed by permethrin, resmethrin and malathion with resistance ratios of 24, 12, and 10, respectively compared to the susceptible S-Lab strain. The Florida strains showed strong tolerance to β-cyfluthrin and may have developed resistance to this insecticide because they were heterozygous depending on its slope. This tolerance was followed by chlorpyrifos. This strain had a slight tolerance to deltamethrin with a resistance ratio of 4.6. It did not develop resistance to fenitrothion. The PR strain had high resistance to the pyrethroid class of insecticide, but it was not resistant to OPs. The reason for this might be the selection of permethrin.

Key Words: *Aedes aegypti*, insecticide resistance

4.2 Introduction

Ae. aegypti, also known as the yellow fever mosquito, is an important vector in the transmission of dengue fever and yellow fever (Brown et al., 2011). *Ae. aegypti* originated from Africa and it has already spread outside of Africa and it has a wide distribution in most tropical and subtropical areas. Parts of Europe, America and Australia are affected (Jansen & Beebe, 2009; Mousson et al., 2005). This species can be transported by trading and transport ships which have

water containers (Jansen & Beebe, 2009; Kraemer et al., 2019). *Ae. aegypti* can choose many habitats because research has shown that this species has a lower infection level in parts of cities that are close to a river, compared with other parts of the city. There is also some evidence show that mosquito density is the highest in medium urban areas and lowest in high density areas. An example is Buenos Aires city (Vezzani & Carbajo, 2008). Due to its outstanding ability to adapt adverse environments it can successfully establish in many places where they have spread. Some studies show that in laboratory conditions, the larvae of this species start to die when water temperatures are over 34 ˚C and adults cannot survive over 40˚C. *Ae. aegypti* from Indian's Thar desert and from northwestern Rajasthan, where temperature usually exceeds 40˚C in the summer, can survive and can transmit dengue because they can find household shelters and underground cement water containers (Jansen & Beebe, 2009). *Ae. aegypti* can survive perfectly in human habitats so this provides a convenient method for them to transmit human disease and in America the urban population doubled from 1970 to 1990 and dengue then became a major health problem (Monath, 1994).

Ae. aegypti acts as a vector for human diseases and it can transmit yellow fever in Central and Southern America and in West Africa. Dengue fever can be transmitted in other areas such as Southeast Asia, the Pacific islands, Africa, and the Americas (Ciccia et al., 2000). In America, the Pan American Health Organization (PAHO) initiated yellow fever eradication in the 1950s and *Ae. aegypti* populations declined in most of Central and South America by the 1970s. However, lately this species has reinfested most areas including North and South America (Jansen & Beebe, 2009). There are studies on the resistance level of *Ae. aegypti* for 6 OPs and 4 pyrethroids that used eight Latin American strains and the results show that some strains had high resistance to one insecticide (Rodríguez et al., 2007).

In Florida, *Ae. aegypti* is mainly located in urban areas (O'Meara et al., 1995). While some studies show the resistance level of *Ae. albopictus* in Florida, but resistance levels of *Ae. aegypti* remains unclear (H. Liu, Cupp, Guo, et al., 2004). In this study, the resistance level of *Ae. aegypti* in the field in Florida was tested and the results were compared between susceptible and resistant strains in the laboratory to understand whether this species has developed resistance in the field.

4.3 Material and Methods

4.3.1 Mosquito Strains

Three strains were used in this study. Two of them came from laboratory strains and one was a susceptible strain. The S-Lab was provided by Dr. Laura Harrington (Cornell University, Ithaca, NY) and the other was a resistant strain, PR. The final strain was collected in the field in Florida. All strains were reared at 25 ± 2°C with a photoperiod of 12:12h (L:D) (H. Liu, Cupp, Micher, et al., 2004).

4.3.2 Insecticides

Eight insecticides were chosen to do bioassays and they had different modes of action. Chlorpyrifos (99.3%), Fenitrothion (95.8%), Etofenprox (99.1%), and β-cyfluthrin (99.9%) were purchased by Sigma-Aldrich®; Malathion (99.2%) and Resmethrin (98%) were bought from Chem Service, Inc.; Deltamethrin (99.9%) and Permethrin (94.34%) were provided by FMC Crop (Princeton NJ).

4.3.3 Bioassay Methods

Acetone was used to prepare a stock solution and different concentration gradients were set up in order to achieve 0% to 100% mortality. Ten 4th instar larvae with similar size were chosen and placed into 20 mL bottles (20 mL disposable scintillation vials VWR®) with 5 mL of tap water. All tests were finished under 25 ± 2°C conditions and results were checked after 24 hours.

Each test was replicated at least three times on different days. Bioassay data were pooled and probit analysis was conducted by using Abbott's correction for control mortality. (H. Liu, Cupp, Micher, et al., 2004). Statistical analysis of LC50 and LC90 values was based on nonoverlap of 95% confidence intervals (CI).

5.4 Result

Ae. aegypti from Florida was resistant to etofenprox with a resistance ratio of 1400 at LC_{50} compared to the susceptible strain S-Lab. *Ae. aegypti* showed lesser resistance to malathion, permethrin and resmethrin with resistance ratios of 11, 24, and 12 at LC50 compared to the susceptible strain. *Ae. aegypti* did not develop resistance to the remaining insecticides but it might develop resistance to β- cyfluthrin because the resistance ratio was borderline at 9.7 at LC_{50} compared with the S-Lab. Deltamethrin was more effective than chlorpyrifos and their resistance ratios were 8 and 5 at LC_{50}, respectively. Fenitrothion had the highest efficacy among these eight insecticides because the resistance ratio was less than 1. Perhaps this strain was selected for permethrin, so it showed high resistance toward pyrethroids but was susceptible to OPs.

For OPs, the field collected strain was homozygous, followed by malathion and chlorpyrifos. The field strain had a similar response to pyrethroids, with slopes that were between 1.4 and 2.4. This strain was homozygous to permethrin compared with other pyrethroid insecticides. Etofenprox had the lowest slope value so this strain was heterogenous to it.

For PR, the resistant strain showed a large difference between pyrethroids and OPs. It showed very high resistance to pyrethroids. The LC_{50} of deltamethrin reached 352ppm and that of β-cyfluthrin reached 654ppm. However, for OPs the LC_{50} was between 0.01ppm and 0.7ppm.

5.5 Discussion

This study showed that *Ae. aegypti* developed resistance to some insecticides including etofenprox, permethrin, and resmethrin. When Florida *Ae. aegypti* developed resistance to one insecticide it was likely to develop resistance to those insecticides with the same mode of action even when they were never exposed to them. In this study *Ae. aegypti* from Florida had a higher tolerance to pyrethroids than OPs. Not only did *Ae. aegypti* from Florida develop resistance, *Ae. aegypti* from other locations also developed resistance. For example, *Ae. aegypti* from Columbia developed resistance to Organochlorines (Ocampo et al., 2011). *Ae. aegypti* from Caribbean islands and neighboring countries showed resistance to temephos, malathion, fenthion, and propoxur (Georghiou et al., 1987). Using newer insecticides was a method to control *Ae. aegypti* but resistance still developed. In Florida, Methoprene has been used for more than three years to control *Ae. albopictus* and it developed tolerance. Insecticides are not only used to control *Ae. aegypti* but also help to control other mosquito species such as *Ae. albopictus* and *Culex quinquefasciatus* (H. Liu, Cupp, Micher, et al., 2004; H. Liu, Cupp, Guo, et al., 2004). Using insecticides can affect various mosquito species and this could promote development of resistance. Insecticides are the main method to control mosquitoes especially the pyrethroid class, which accounts for 25% of the world insecticide market. This class is the only one recommended for mosquito net treatment, which is the primary method of malaria control in Africa (N. Liu et al., 2006; Nkya et al., 2013). Pyrethroid insecticides target the sodium channel and can have a rapid knock-down effect by disrupting the electrical signal in the nervous system (Hemingway et al., 2004; Soderlund, 2010). The development of resistance to pyrethroids can be caused by mutations in the target site and this mutation can be referred to as a knock-down resistance (Nkya et al., 2013). This kind of mutation can be selected and can be developed by cross resistance. DDT, for

example, has the same mode of action as pyrethroids (Nkya et al., 2013). Ops are also widely used with a different mode of action (Kwong, 2002). Ops work on AChE resulting in accumulation of neurotransmitters that continuouly stimulate acetylcholine receptors (Kwong, 2002). Resistance of OPs can be caused by increased esterase activity and insecticide-insensitivity to AChE (Vaughan et al., 1998).

The result of PR showed a big difference between pyrethroids and OPs. These agents have different modes of action. Pyrethroids work at sodium channels, which are important in the depolarization phase of action potentials. The resistance of pyrethroids can be divided into two important groups, one is metabolic detoxification and the other is target site insensitivity (N. Liu et al., 2006). P450s, hydrolases, and GSTs are major enzymes which are involved in metabolic detoxification. The P450s are the most important one among these three enzymes (N. Liu et al., 2006; Scott, 1999). The modification of sodium channels can cause resistance because of the reduced sensitivity to sodium channels and the term “knockdown resistance” is used to describe cases of pyrethroid resistance (N. Liu et al., 2006). The target of OPs is AChE, which is responsible for the hydrolysis of acetylcholine, a neurotransmitter in the nervous system and the modification of which can lead to resistance (H. Liu et al., 2005).

Overuse of insecticides can lead to some negative effects such as the reduction of the density of wild bees, toxicity to fish, and the poisoning of birds (Haya, 1989; Mendelssohn & Paz, 1977; Rundlöf et al., 2015). Finding newer methods of control is important. Newer chemicals are used to control mosquitoes such as insect growth regulators (IGR), naturalytes, biolarvicides and bacteria (Marcombe et al., 2014). One study tested three classes of IGRs, ecdysone agonist, chitin synthesis inhibitor and juvenile hormone analog. Results showed that egg the hatching rate of *Ae. aegypti* decreased (Suman et al., 2013). Biolarvicides are usually extracted from plants and they

can impact pest behavior, however, they have some negative side effects like being biodegradable (Dias & Moraes, 2014). Therefore, using multiple methods is important to control mosquitoes and can help to delay the development of resistance. Understanding resistance mechanisms is important in the control of mosquitoes especially when insecticide resistance occurs. Using insecticides wisely and correctly can help to delay the process of resistance development.

Table 2 Toxicity of different insecticides to *Ae. a*egypti from Florida compared with S-Lab and PR strains

Insecticides	Strains	DF	$\chi 2$	n	$LC_{50}{}^{b}(CI)^{c}$	$LC_{90}{}^{b}(CI)^{c}$	Slope Values
Chlorpyrifos	FL	5	3.6	175	0.04(0.03,0.05)	0.09(0.07,0.2)	3.0(0.4)
	S-Lab	3	1.7	200	0.005(0.004,0.006)	0.02(0.009,0.02)	3.1(0.4)
	PR	2	2.6	253	0.03(0.02,0.05)	0.07(0.05,0.5)	3.2(0.4)
Fenitrothion	FL	6	6.9	175	0.03(0.03,0.04)	0.07(0.05,0.1)	4.2(0.7)
	S-Lab	3	1.3	199	0.10(0.008,0.01)	0.03(0.02,0.05)	2.9(0.5)
	PR	3	0.0	176	0.02(0.015,0.021)	0.03(0.03,0.04)	5.7(0.8)
Malathion	FL	5	6.8	188	2.3(1.8,3.1)	4.9(3.6,9.9)	4.0(0.6)
	S-Lab	3	0.0452	190	0.2(0.2,0.3)	0.4(0.3,0.6)	4.7(0.7)
	PR	2	2.5	233	0.28(0.2,0.4)	0.6(0.4,3.3)	3.6(0.5)
β-cyfluthrin	FL	4	7.2	257	0.014(0.008,0.022)	0.07(0.04,0.2)	1.9(0.2)
	S-Lab	4	0.0024	238	0.0015(0.0013,0.0017)	0.003(0.002,0.003)	5.6(0.7)
	PR	3	5.8	277	654.1	31545	0.8(0.3)
Deltamethrin	FL	4	8.7	248	0.009(0.005,0.014)	0.04(0.02,0.12)	2.0(0.2)
	S-Lab	3	0.4	279	0.002(0.0016,0.0022)	0.005(0.004,0.007)	3.1(0.3)
	PR	3	2.2	159	352	888428.6	0.4(0.1)
Permethrin	FL	7	9.3	250	0.5(0.4,0.7)	1.8(1.2,3.5)	2.4(0.3)
	S-Lab	4	5.4	450	0.02(0.02,0.03)	0.06(0.04,0.1)	3.0(0.3)
	PR	3	1.4	152	2387.7	2549100	0.6(0.3)
Resmethrin	FL	3	0.4	169	0.6(0.4,0.8)	2.7(1.8,5.0)	1.9(0.2)
	S-Lab	2	0.0	236	0.05(0.04,0.05)	0.08(0.07,0.1)	5.3(0.7)
	PR	2	1.1	223	1211100	8764500000	0.3(0.2)

4.4 Reference

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Chapter 5 Comparison P450 Expression Level of *Aedes aegypti* from Florida with Susceptible and Resistant Strains

5.1 Abstract

The P450 enzymes are involved in many important metabolic functions including metabolism of foreign chemicals such as insecticides, environmental pollution, and plant toxins. They are also involved in the synthesis of endogenous compounds such as juvenile hormone and ecdysteroid. CYP4, CYP6 and CYP9 are important for the metabolism of insecticides so in this experiment the CYP4H30 and CYP6CB1 genes were chosen for analysis of their expression level. CYP4H30 showed no difference among AeFl, susceptible S-Lab, and resistant PR strains. CYP6CB1 showed high resistance in PR strains with an expression ratio of 33.3 and showed up-regulation in AeFl with a ratio of 5.6.

Key Words: *Aedes aegypti*, P450 genes, qRT-PCR

5.2 Introduction

Cytochrome P450 proteins are one of the largest group of enzymes and genes that encode them can be found in all organisms including plants, prokaryotes, and eukaryotes (Werck-Reichhart & Feyereisen, 2000). They have great importance and are of high interest due to their numerous biological functions such as degradation of drugs, environmental chemicals, and their biosynthesis of endogenous compounds (Rendic & Carlo, 1997). But for different P450s, the range of substrates is quite different. CYP1A1 can metabolize more than 20 substrates while CYP7A1 can only recognize a single substrate (Scott, 1999).

Total P450 was first detected in an insect in 1967, and more than 100 insect P450s have since been discovered (Scott, 1999). In insects P450s can be found in the midgut, fat body, and Malpighian tubes. Midgut P450s typically have the highest activity (Hodgson, 1983). The period of occurrence of P450s can vary, in general total P450s cannot be detected in the egg stage. They rise and fall in each larval stage. They are undetectable in the pupa stage and they will increase in the adult stage (Scott, 1999). Some insect P450s can be specific in one stage or can be life stage independent (Scott et al., 1998). P450s play an important role in metabolic system. They can catalyze degradation of exogenous compounds like insecticides and plant toxins. They also play a role in the synthesis of endogenous compounds that are important in insect growth, development, and reproduction (i.e., pheromones, juvenile hormone, and 20-hydroxyecdysone) (N. Liu et al., 2015).

P450s are important for the degradation of xenobiotics and endogenous compounds while increased levels of enzyme activity can lead to insecticide resistance that is an obstacle in pest management (N. Liu, 2015). When resistance occurs, increasing the dose and application frequency does not help to reduce the economic losses and may increase human health risk (Pimentel & Burgess, 2014). A study involved in the resistance level of *Culex quinquefasciatus* from Florida showed that there was resistance and cross-resistance in this species. The resistance level reached up to 200-fold and 830-fold for resmethrin (H. Liu et al., 2004). Resistance among *Ae. aegypti* remains unclear. It is important to test the resistance level of this species and to understand the resistance mechanism. These findings will be helpful for the wise selection of insecticides.

5.3 Material and Methods

5.3.1 Mosquito Strains

The strain of AeFl of *Ae. aegypti* was collected in Florida and was reared in a laboratory. Two laboratory strains, one susceptible strain, S-Lab, was provided by Dr. Laura Harrington (Cornell University, Ithaca, NY), and the other was a resistant strain, PR. All strains were reared under at 25 ± 2°C with a photoperiod of 12:12h (L:D) (H. Liu et al., 2004).

5.3.2 Quantitative real-time PCR (qRT-PCR)

Twenty female adults from different generations were selected for qRT-PCR and RNA extracted for each experiment by using the acidic guanidine thiocyanate-phenol-chloroform method. Total RNA (1 µg / sample) from each mosquito sample was reverse transcribed using SuperScript II reverse transcriptase (Thermo Fisher Scientific) in a total volume of 20 µL. Before qRT-PCR, the concentration of each sample was measured by using NanoDrop. qRT-PCR was performed by using SYBR green master mix Kit and ABI 7500 Real Time PCR system (Applied Biosystems). Each qRT -PCR contained 5 µL SYBR Green master mix, 1 µg cDNA, 0.5 µl forward and reverse primers separately adding ddH_20 up to 10 µl. The reaction cycle consisted of a denaturation step of 98 °C for 30 seconds, followed by 40 cycles of 98 °C for 15 seconds and 60 °C for 30 seconds. All experiments including reference was repeated at least three times.

6.4 Result and Discussion

CYP4H30 and CYP6CB1 genes were chosen to analyze their expression level. The CYP4H30 gene showed up-regulation in PR but no change in AeFl. The CYP6CB1 gene also showed up-regulation in PR, but it showed down regulation in AeFl.

Some studies showed that P450 gene over expression was involved in resistant CYP4 and CYP9 (Bariami et al., 2012; Strode et al., 2008). Some others have focused on the functional study

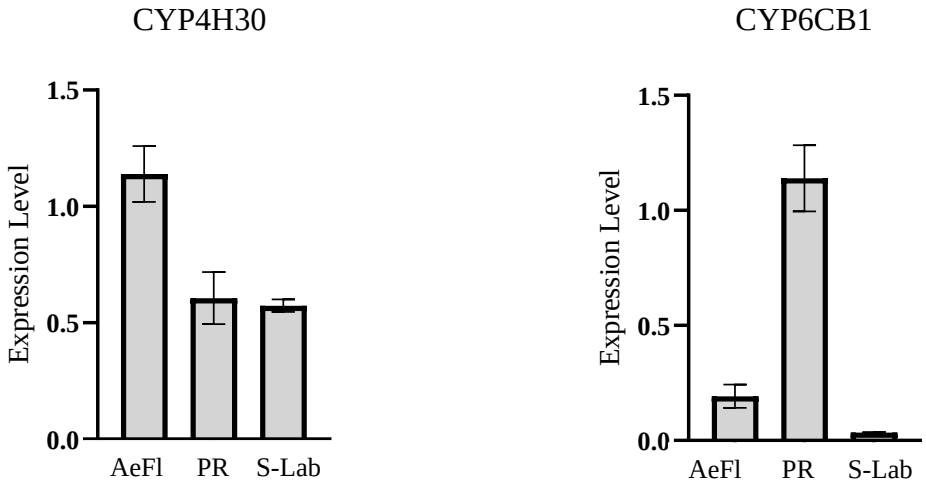
of insect cytochrome P450s especially in CYP6 and CYP9, but information related with pyrethroid resistance is still sparse (Reid et al., 2014). According to previous study, the CYP4H30 and CYP6CB1 genes are upregulated in *Ae. aegypti* from Puerto Rico, these same genes were analyzed in this experiment (Reid et al., 2014).

CYP4 is the one of the oldest P450 families, containing 22 subfamilies (Simpson, 1997). Subfamily A and B were identified in animals including rats, humans, and rabbits. In subfamily C, some genes were identified in the *Blaberus discoidalis* cockroach, and CYP4H was identified in *Anopheles albimanus* mosquitoes (Simpson, 1997). The CYP6 family is involved in the metabolism of insecticides in insects (Zhang et al., 2019). In *Anopheles gambiae* permethrin and deltamethrin can be metabolized by YP6P3 and CYP6M2 (Zhang et al., 2019).

The P450 enzymes play an important role in the synthesis of hormones and the degradation of foreign chemicals. Insect genomes might carry 100 P450 genes coding for different P450 enzymes (Feyereisen, 1999). P450 genes have a complicated regulatory process, with induction playing an important role in adaptation to plant chemicals, and mutations playing a central role in insecticide resistance (Feyereisen, 1999). Different P450s have different functions and some of them have life stage specificity. The CYP4D1 and CYP6A1 genes can express at all life stages but the CYP6D1 gene is adult specific, and CYP6B2 is larval specific (Scott et al., 1998). Increased enzyme activation is the most common resistance mechanism and P450 enzymes mediated resistance might be the most frequent type of metabolism (Scott, 1999). In some resistant insects, some P450 genes can express at a high level. In *Anopheles gambiae* the resistant strain had a high expression level for CYP4C27, CYP4H15, CYP6Z1, CYP6Z2, and CYP12F1 (Daborn et al., 2001). CYP6P9 and CYP6P4 showed increased expression in pyrethroid resistance in the

Anopheles funestus strain (Wondji et al., 2009). Therefore, identification of new cytochrome P450 genes is an important step in the management of mosquitoes.

Figure 3 The expression level of two P450 genes, CYP4H30 and CYP6CB1



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Chapter 6: Future study

Mosquitoes can transmit several human diseases and may lead to death. Management of mosquitoes mainly depends on insecticides, overuse of which can contribute to resistance. There are several resistance mechanisms including metabolic mechanism, target insensitivity, physiological modification and behavioral resistance. Among these mechanisms metabolic mechanism is the most common one that occurs in insects (Corbel & N'Guessan, 2013). Over-expression of enzymes, which can detoxify insecticides, or substation of amino acids in these enzymes can lead to high resistance. Increased expression of genes encoding these enzymes is the major mechanism of resistance (Corbel & N'Guessan, 2013). Cytochrome P450 enzymes are important in the metabolism of insecticides and currently the P450 gene superfamily comprises 70 families with 127 subfamilies (Scott, 1999). In mosquitoes CYP4, CYP6 and CYP9 are found to increase transcription and are involved in various insecticide resistance (Corbel & N'Guessan, 2013). In my research, I focused on the CYP4H30 and CYP6CB1 genes, and I compared the expression level in three strains of mosquitoes.

There are more than 660 insect P450 genes involved in resistance and methods such as the comparison of gene sequences, copy number, and expression levels can be used to determine the molecular mechanisms of P450-mediated resistance (Li et al., 2007). Gene amplification can cause resistance and there are 25 P450 genes belonging to family CYP4, CYP6 and CYP9 that can be overproduced by upregulation (Li et al., 2007). Another type of resistance can be caused by coding sequence changes (Li et al., 2007). Point mutations may play a secondary role in resistance and there is some evidence that amino acid substitution occurs in P450 genes including CYP6X1, CYP6D1, CYP6D3, and CYP6A2 (Li et al., 2007). When insects develop resistance to different insecticides various genes are involved. The CYP6G1 gene is involved in DDT resistance in

Drosophila, and the CYP6P9 and CYP6P4 genes are involved in pyrethroid resistance in *Anopheles funestus* (Daborn et al., 2001; Wondji et al., 2009).

In the future, I also wish to focus on P450 genes in two parts. One part is to discover which genes are overexpressed for resistance. Bioassays can help us to understand if filed mosquitoes develop resistance to insecticides. If insecticides are developed using molecular technology, then we can find specific genes related to resistance. The second part is involved in finding genes that are related to insecticide resistance. This part will mainly focus on finding genes that correspond to a single insecticide.

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