Aedes Mosquitoes in Alabama – Population Dynamics and Sensitivity to Insecticides

by

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Abstract

The Asian tiger mosquito, *Aedes albopictus*, is one of the most notorious day-biting species of mosquito in the world and can transmit approximately 26 diseases, including dengue, of which more than 50 million cases occur each year; chikungunya, which has spread to 40 countries located in Asia, Africa, Europe, and America and caused more than 1,500,000 cases in 2006; and yellow fever, which has caused several serious outbreaks in Bolivia, Peru, Ecuador, Brazil, and Africa. Insecticides are still the most efficient way to combat and prevent mosquito-borne diseases and control mosquito populations among several approaches. However, intensive and inappropriate use of insecticide results in insecticide resistance, which is a major barrier to the development of a management strategy to control mosquito populations. Plenty of papers have reported insecticide resistance in different mosquito species, and *Aedes aegypti* populations from around the world have been confirmed be resistant to a variety of insecticides, such as temephos, malathion, deltamethrin, permethrin, DDT and propoxur. *Aedes albopictus*, which is less resistant than *Aedes aegypti*, is still resistant to several insecticides, such as chlorpyrifos, pyrethroids, and carbamates. Insensitive targets, the overexpression of detoxifying enzymes, penetration resistance, behavioral avoidance, and so on have been confirmed to play roles in insecticide resistance. Mutations can change the properties of the target site, affecting insecticide binding to the target, and gene overexpression of detoxifying enzymes can generate more detoxifying enzymes to degrade more toxins. Surveillance of the mosquito population and insecticide resistance are fundamental for controlling mosquito-borne diseases and could be used by the government to optimize the strategy to fight diseases transferred by mosquitoes.

In this research, traps used to collect mosquito eggs were placed in several locations scattered across Alabama and were checked every two or three weeks from July 2017 to July 2018. Three species were identified in this survey in Alabama: *Aedes albopictus*, *Aedes triseriatus*, and *Aedes japonicus*. The mosquito population began to emerge in April at approximately 57°F. The suitable temperature range of mosquito is 75°F to 85°F. *Aedes albopictus* was tested for its resistance level to eight insecticides, chlorpyrifos, deltamethrin, etofenprox, malathion, permethrin resmethrin, fenitrothion, and β-cyfluthrin, in adult and larval bioassays. Resmethrin, permethrin, and fenitrothion were found to allow the mosquitoes to survive longer than the diagnostic time. Mobile is the most suitable city for mosquitoes, with most strains demonstrating insecticide resistance. We suggest that β-cyfluthrin and deltamethrin should replace permethrin and malathion for controlling mosquitoes. Altogether, our study data provide fundamental information for the government to establish a strategy for controlling mosquitoes in the future.

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ITN	Insecticide Treated bed Nets
IRAC	Insecticide Resistance Action Committee
OP	Organophosphate
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChR	Acetylcholine Receptor
GABA	γ-Aminobutyric acid
GST	Glutathione S-Transferases
CYP	Cytochrome P450
Rdl	Resistance to dieldrin
RNAi	RNA interference
GAM	Generalized Additive Model
GPCR	G Protein-Coupled Receptors
ZFN	Zinc-Finger Nucleases
TALENs	Transcription Activator-Like-Effector Nucleases
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CDC	Centers for Disease Control and Prevention
OD	Optical Density
WNV	West Nile Virus

Chapter 1: Literature Review

1.1 Problems caused by mosquitoes

The mosquito, one of the most annoying insects in the world, occurs worldwide and affects the lives of billions of people. The mosquito can not only make people uncomfortable due to biting and noise but can also transfer viruses, thereby causing human illness and even death. Moreover, some of these diseases can be catastrophic. Malaria, which is one of the most notorious diseases in history, can be traced back for more than 2,000 years and has caused great suffering in many people in generation after generation (Kidson and Indaratna, 1998); malaria is mainly transmitted by *Anopheles,* and this determination by Sir Ronald Ross represents a milestone in the history of malaria. According to a report from 2012, nearly 207 million cases of malaria occurred; these cases killed nearly 482,000 children under five years old; and in every minute, a child dies of malaria. Dengue, which contributes to more than 50 million cases each year, causes more than 2.5 billion people who dwell in dengue-endemic countries to suffer (Organization, 2009). Importantly, dengue fever has indicated a potential to resurrect in recent years (Messina et al., 2014). Unlike malaria, dengue is transferred by *Aedes aegypti* and *Aedes albopictus* (Bhatt et al., 2013; Simmons et al., 2012). Another virus transferred by mosquitoes should also be highlighted: chikungunya, typically a tropical illness, which has spread through more than 40 countries in Asia, Africa, Europe, and America. Recently, an outbreak in India caused more than 1,500,000 cases in 2006: 68% of these cases were in women, and 74% of the victims were older than 30 years of age, which means adults and women were more affected than others (Pialoux et al., 2007). Chikungunya has the same carrier mosquitoes as dengue (Staples et al., 2009). Finally, yellow fever, which was effectively controlled in the mid-19th century but has been resurrected in the last 25 years of the 20th century, infects nearly 200,000 people annually. According to some articles, several serious outbursts have occurred in Bolivia, Peru, Ecuador, Brazil and Africa (Barnett, 2007; Gubler, 2004). *Aedes aegypti*, as the most notorious mosquito species, transmits this disease (Monath, 2001).

The most popular disease transferred by mosquitoes recently is Zika virus, which was added to the Public Health Emergency of International Concern on 1 February 2016 (Organization, 2016c), causing irregular disease in Africa, Asia, and America. Zika virus was first reported in 2007 and 2008, with 185 confirmed cases on Yap Island. According to Brazilian national authorities, approximately 500,000 to 1,500,000 cases of Zika virus disease have been reported. Not only Brazil but also Colombia and Cabo Verde have reported outbreaks of Zika virus. Up to 19 March 2016, Colombia had reported 56,477 suspected cases of Zika virus, whereas Cabo Verde had reported 7,499 suspected cases as of 6 March 2016 (Organization, 2016b, d). Unfortunately, *Aedes albopictus* and *Aedes aegypti* have been identified as spreading this disease (Plourde and Bloch, 2016).

Aedes albopictus and *Culex quinquefasciatus* are two widespread mosquito species in Alabama, and they can transmit several serious diseases. The diseases transmitted by *Aedes albopictus* not only include three (dengue, Zika, and chikungunya) of the five famous diseases mentioned above but have the potential ability to transfer more than 26 viruses according to experiments with the *Flaviviridae* (genus *Flavivirus*), *Togaviridae* (genus *Alphavirus*), *Bunyaviridae* (genus *Bunyavirus* and *Phlebovirus*), *Reoviridae* (genus *Orbivirus*) and *Nodaviridae* (genus *Picornavirus*) families (Paupy et al., 2009). *Aedes albopictus* is responsible for outbreaks that have occurred recently for several diseases. For example, it is the only or the main vector of the outbreak in 2005-2007 of chikungunya, which occurred in all of the Indian Ocean islands and surrounding countries (Madagascar, Mauritius, Maldives, India, Indonesia, and Malaysia Madagascar), and this was later confirmed as the main vector of this disease outbreak during the first European outbreak reported in Italy in August 2007 (Angelini et al., 2008; Paupy et al., 2009; Paupy et al., 2010); in addition, it was the primary vector for chikungunya in the Gabonese outbreak according to Entomological surveys (Paupy et al., 2010). *Aedes albopictus* is also a major vector of arboviruses responsible for several outbreaks of disease.

As a member of the *Culex pipiens* complex, *Culex quinquefasciatus* can transfer various diseases that have caused dramatic outbreaks in the world, e.g., the West Nile virus, St. Louis encephalitis in the eastern United States, periodic lymphatic filariasis (*Wuchereria bancrofti*) (Farid et al., 2001), avian malaria (Fonseca et al., 2000), and other encephalitis diseases around the world. The control of all these diseases is critical (Smith and Fonseca, 2004). West Nile virus (WNV), a disease transmitted between birds and mosquitoes and that infects humans, horses and other mammals, has an extensive distribution throughout Asia, the Middle East, southern Europe, western Russia, southwestern Asia and Australia (Petersen et al., 2013). West Nile virus was first introduced into the United States in 1999 (Savage et al., 2007). Between 1999 and 2012, West Nile virus affected 16,169 patients and led to 1,549 deaths, and it has now spread everywhere in the United States except Alaska and Hawaii (Petersen et al., 2013). Compared to the United States, West Nile virus was first detected in Southern Ontario in 2001, and in 2009, the distribution of West Nile virus had expanded to British Columbia. As of October 2012, 975 patients with West Nile virus had been reported in Canada (Petersen et al., 2013). Another disease transferred by *Culex quinquefasciatus* should also be mentioned—Japanese encephalitis; this vector-borne disease, occurs in both Asia and Pacific. Approximately 3 billion people live in countries where the Japanese encephalitis virus is endemic, and the annual incidence of this disease is 30,000-50,000 cases (Erlanger et al., 2009). Most adults in these areas are already immune to the Japanese encephalitis, so children under 15 years of age are the primary victims of this disease (Lopez et al., 2015)

1.2 *Aedes* and *Culex*

Both *Aedes albopictus* and *Culex quinquefasciatus* can transfer various and dangerous diseases and viruses in the world. *Aedes albopictus* is a primary vector of dengue, malaria, chikungunya, yellow fever, and Zika fever; *Culex quinquefasciatus* primarily spreads West Nile virus, St. Louis encephalitis, filariasis, and avian malaria. Both mosquitoes belong to the order *Diptera*, the family *Culicidae,* and the subfamily *Culicinae*, but they come from different genera – *Aedes albopictus* belongs to genus *Aedes* genus, and *Culex quinquefasciatus* belongs to the genus *Culex* genus and the species complex *Culex pipiens*.

Aedes albopictus, one of the most notorious day-biting mosquito species in the world, can transmit approximately 26 viruses and is responsible for various outbreaks in history. *Aedes albopictus* mainly came from Asia and spread west to the island in the Indian Ocean, and east to the island in the Pacific Ocean during the 19th century and the early 20th century (Knudsen, 1995). However, no *Aedes albopictus* movement was reported in the subsequent 20 years. Since the 1980s, however, a large-scale migration of *Aedes albopictus* has been reported (Benedict et al., 2007): in Albania in 1979 (Adhami and Reiter, 1998), in Texas in 1985 (Lambrechts et al., 2010), in Brazil in 1986 (Gratz, 2004), among others. Subsequent to that, in the following two years, *Aedes albopictus* became established in several countries, including from the United States to Argentina, Central Africa, Europe, Australia, among others. (Gratz, 2004; Paupy et al., 2009). As for the United States, the first report of *Aedes albopictus* was from 1985 in Texas, and since that time, this species recently has become established in 866 counties in 26 states (Bonizzoni et al., 2013; Lambrechts et al., 2010). The used tire industry is responsible for the distribution of *Aedes albopictus*. As the tires were shipped to the rest of the country, *Aedes albopictus* was also transferred to other places (Reiter, 1998). Until now, *Aedes* still has the ability to spread around the world, which indicates that disease related to *Aedes* poses a potential risk for an outbreak (Lambrechts et al., 2010). Furthermore, the distribution of *Aedes albopictus,* which is occurring mainly in tropical and subtropical areas, can also spread to the rest of the world through shipping.

In 1832, *Culex quinquefasciatus* was first recognized as belonging to the *Culex pipiens* complex (Fonseca et al., 2004; Ward, 1984), and like other members in the species complex, *Culex quinquefasciatus* is seldom found far from human residence or activity and readily feeds on avian, mammalian or human hosts as a peridomestic mosquito (Farajollahi et al., 2011). *Culex quinquefasciatus* thrives in tropical and subtropical regions, including those in the African lowlands, Americas, Asia, and Australia (Fonseca et al., 2006). Like other species that spread worldwide through shipping, the Atlantic slave trade, Old China trade, and the American whale oil industry played major roles in the transportation of *Culex quinquefasciatus* between the 17th and 19th centuries (Lounibos, 2002)

1.2.1 Morphology of *Aedes* and *Culex*

Adult *Aedes* mosquitoes have a narrow and representative black body, distinctive patterns of light and dark scales on the abdomen and thorax, alternating light and dark bands on the legs, and a slightly curved or straight proboscis, which are used to distinguish this species from other species of mosquitoes (Azari-Hamidian and Harbach, 2009).

Culex adults are usually drab, concolored mosquitoes, but some species of the subgenus *Culex* share characteristics on their legs and pale spots on their wings with *Anopheles.* The major way to distinguish *Culex* from other mosquitoes is by observing the flagellomere 1 is as long as flagellomere 2 and the absence of prespiracular setae (Azari-Hamidian and Harbach, 2009).

1.2.2 Life cycle of mosquitoes

Mosquitoes, like other holometabolous insects, go through four complete stages: eggs-larvae-pupae-adults, usually taking 8-10 days from eggs to adult, and you can see them both inside and outside of the home (**Figure 1**).

Female mosquitoes lay their eggs on almost any places where they can find a little stored water, such as in bottles, bowls, tree holes, or flower pots. Female mosquitoes can lay as many as 100 to 200 eggs in each clutch and can produce five clutches in their lifetime. The eggs, which are smooth, long, and ovoid shaped, can usually survive 8 months when overwintering in the southern United States.

The larvae usually float on the surface of the water and survive by eating microorganisms or other organic matter, while growing from the first until the fourth instar (males developing faster than females). The larvae then change to the pupal stage.

Pupae are mobile and respond to stimuli, but they do not feed, eventually changing into an adult and leaving the water. Adult male mosquitoes feed on nectar, and adult females feed on blood from animals or humans.

1.2.3 Habits of *Aedes* and *Culex*

Aedes is a painful and persistent biter, usually active in the daytime, especially early in the morning and during the twilight before sunset. Some are diurnal (daytime biters) and prefer to hang out during cloudy days or in shaded areas. Notably, *Aedes* prefers to feed on humans to other animals, so it usually emerges near residential areas. *Aedes* mosquitoes are strong fliers and are known to fly many miles from their larval development sites.

Similar to *Aedes*, *Culex* is a painful and persistent biter, but prefers to attack at dusk and after dark. *Culex* also enters dwellings for blood meals, unlike *Aedes,* which is more active near the residential area. Comparatively, *Culex* mosquitoes are generally weak fliers and do not move far from home, although they have been known to fly up to two miles. *Culex* lives for only a few weeks during the warm summer months. Those females that emerge in the late summer search for sheltered areas where they can hibernate until spring. Warm weather brings them out again in search of water on which to lay their eggs.

1.3 Mosquitoes control

To prevent and reduce the transmission of the mosquito-borne diseases (dengue, chikungunya, yellow fever, and Zika virus), the most effective methods are to control the population of the mosquitoes and cut off the connection between human and mosquitoes. Four major methods exist to control the mosquitoes: environmental, biological, mechanical, and chemical.

1.3.1 Environmental, biological, and mechanical control

Developing maps on the distribution of mosquitoes and discouraging people from occupying places where mosquitoes are highly concentrated could be an efficient method to decrease the morbidity from mosquito vector-borne diseases. In addition, altering the conditions of areas mosquitoes could use for laying eggs is another valid method. As for permanent containers, the use of a nylon net or other tight-fitting overlapping cover for the water surface could be the main approach. Covering the surface of water could prevent the female mosquitoes from touching the water and laying eggs, and tightly fitted tops could allow the rainwater to be harvested from roofs through mesh screens, while keeping mosquitoes out most of the time. As for temporary water containers, removing them or dumping water from them could be a better idea. One must be careful of those places where rainwater could easily be stored (Baldacchino et al., 2015). On the other hand, traps, which are widely used for the survey and monitoring of mosquito populations, have also been suggested as an approach for reducing adult populations of mosquitoes (Baldacchino et al., 2015).

Natural enemies have also been used as an efficient method for controlling mosquitoes, and some species can kill more than 40 mosquitoes per day. Residents could keep and feed fish in containers to kill the larval of mosquitoes (Chandra et al., 2008; Martinez-Ibarra et al., 2002). Copepods, another predator for mosquitoes, can be used as an efficient approach against mosquitoes (Lazaro et al., 2015). Fungi such as *Beauveria bassiana* and *Metarhizium anisopliae* also provide an efficient way to control mosquito numbers (Baldacchino et al., 2015). Except for *B. thuringiensis* and *B. sphaericus*, other species used for biological control have been found capable of killing mosquitoes (De Barjac and Sutherland, 2012; Scholte et al., 2004).

1.3.2 Chemical control

Compared to other mosquito-control methods, the use of insecticides is still the widest-used approach because insecticides can reduce mosquito populations rapidly. Larvicides could be used directly in a water-storage container; they have low toxicity to other species and cause no significant changes in the color, the quality or the taste of the water. Insecticide-treated bed nets (ITNs) are mosquito nets treated with insecticide and also provide a major component of mosquito control by providing personal protection and reducing the number of mosquitoes in the nearby area (Strode et al., 2014).

According to IRAC (Insecticide Resistance Action Committee), insecticides have been divided into 5 classes and 30 groups based on their target system and mode of action: insecticides, such as carbamate, primarily target nerve and muscle systems; organophosphate focuses on mosquito acetylcholinesterase; organochlorines and fipronil act against the GABA-Gated chloride channel; DDT and pyrethroids target the sodium channel; neonicotinoids and nicotine target the nicotinic acetylcholine receptor, and so on. Insect development is another target system that could be used to kill pests: Analogs can mimic juvenile hormone and disturb insect development. Cellular respiration can also be targeted by diafenthiuron, which inhibits ATP synthase in the mitochondria. The midgut, as an important organ in the digestive system. could be targeted by *Bacillus*, which secretes microbial toxins that destroy the membrane. The last class of insecticides, which includes sulfur, chloropicrin, and borates, has an unknown target or multiple targets (Sparks and Nauen, 2015).

Among these pesticides, the most widely used are organophosphates and pyrethroid. In fact, 262 tons per year of organophosphate (OP) insecticides has been utilized, and 39 tons per year of pyrethroids has been utilized (Manjarres-Suarez and Olivero-Verbel, 2013; Zaim et al., 2007). People use them to spray indoors and in other places near the residence where the potential exists for mosquito breeding (Baldacchino et al., 2015).

However, two new problems have emerged along with the wide overuse of insecticide: insecticide resistance has become the most serious obstacle to pesticide control of these pests, and the other obstacle is the limited number of new insecticides being commercialized for vector control (Liu, 2015).

1.4 Insecticide resistance in mosquitoes

Mosquitoes have developed some ingenious strategies against the insecticide toxicity, which have been called insecticide resistance. Even worse, the greater the resistance ability of the mosquitoes, the more widespread mosquito-borne diseases will be (Smith et al., 2016). The resistance of mosquitoes has been reported widely in plenty of locations, such as Brazil, Cuba, Venezuela, the Caribbean area, China and the United States, among others (Da-Cunha et al., 2005; Georghiou et al., 1987; Harris et al., 2010; Hemingway et al., 1989; Liu et al., 2004b; Maciel-de-Freitas et al., 2014; Macoris et al., 2007). Insecticide resistance was first recognized by scientists in the 1950s when *Aedes* and *Culex* populations in Florida were able to resist DDT larvicide (Giullin and Peters, 1952). *Aedes albopictus* has been confirmed to resist insecticides in China, India, Malaysia, Thailand, Brazil, the United States, and Italy; the highest resistance was observed in 1991in China for DDT (LC50=13.94 ppm). Furthermore, the temephos resistance status of this species varies in different locations (LC50s 0.003~0.048); it was lowest in 1991 in China and in 2007 from Cameroon, with the highest values reported in 2009 from Greece and Italy (Vontas et al., 2012). Another species like *Anopheles*, which is also a species that should not be shown mercy, can transfer various diseases and can be detected to have a strong resistance to insecticides in 78% of the countries that reported monitoring data (Hemingway, 2014; Mnzava et al., 2015). Insecticide resistance not only included a single strain to one type class of insecticide, it also contains multiple-resistance, which is when pests show generalized resistance to more than one class of pesticide. The cross resistance, which is a genetic mutation phenomenon, makes the pests resistant to more than one pesticide with similar mechanisms of action (Daly et al., 1978). The various types of resistance make the mechanism of resistance more sophisticated and cramped.

Figuring out the mechanism of insecticide resistance is the first and the most important step for control; after that, scientists could create some new and more effective strategies to prevent the resistance, control the resistance in mosquitoes, and eventually reduce the popularity of mosquito-borne disease (Liu et al., 2015; Xu et al., 2005).

1.4.1 How the resistance emerges

Many mosquitoes are killed when an insecticide is used the first time; however, a small number of mosquitoes that carry a resistance gene survive and transfer the gene to the next generation. After long-term use and overuse of one class of insecticide, that number of mosquitoes that carry the resistance gene becomes larger and larger, with the insecticide resistance developed, making the insecticide worthless (Liu et al., 2015; Smith et al., 2016).

1.4.2 Resistance of *Aedes aegypti* and *Aedes albopictus*

Aedes aegypti has been confirmed to be resistant to a variety of insecticides, including temephos, malathion, deltamethrin, permethrin, DDT, chlorpyrifos and propoxur, worldwide (Chen et al., 2016; Kamgang et al., 2017; Li et al., 2018; Liu et al., 2004a; Marcombe et al., 2014; Ponlawat et al., 2005). However, *Aedes albopictus* is still less resistant than *Aedes aegypti*, which has been reported to be significantly resistant to 3 major classes of insecticides: chlorpyrifos, pyrethroid, and carbamates worldwide (Goindin et al., 2017; Ishak et al., 2015; Li et al., 2015; Marcombe et al., 2014; Ponlawat et al., 2005; Rodríguez et al., 2007; Seixas et al., 2017).

1.4.3 Mechanism of resistance

More than 1500 published research papers have addressed the mechanisms of insecticide resistance and its development in mosquitoes (Liu, 2015). Some have said a gene mutation could be responsible for the resistance, e.g., L-F and H-S mutations in the sodium channel (Dong, 2007); some thought the overexpression of enzymes led to the resistance (Casida and Durkin, 2013; Hemingway and Ranson, 2000; Hemingway et al., 2000; Liu et al., 2015); some papers reported that the decreasing absorption of insecticide is linked to the insecticide resistance (Plapp, 1976); and some papers reported that behavioral avoidance by the mosquitoes to insecticide also plays a key role in insecticide resistance (Chareonviriyaphap et al., 2013). Altogether, the insensitivity of the target and the increase in insecticide metabolism are two particular mechanisms of insecticide that have been intensively studied, and their importance also has been widely acknowledged by most scientists (Ranson et al., 2011).

1.4.3.1 Target site of insecticide

Some insecticides, such as DDT and pyrethroids, target the sodium channel, which can slow or totally hinder the sodium channel and prolong inactivation, eventually killing the pests (Gilbert and Gill, 2010). The other target is used by OP and carbamate-acetylcholinesterase, which hydrolyze acetylcholine neurotransmitters and end the transfer of signals, thereby exterminating the pests (Fournier and Mutero, 1994). Furthermore, the γ-aminobutyric acid receptors (GABA) which are targeted by cyclodiene and fipronil are also another key target for insecticides (Cole et al., 1993).

The toxic and insecticidal effect of pyrethroid and DDT occurs by prolonging the open state of the sodium channel for an unusual time and inhibiting the nerve repolarization, making insects die from exhaustion (Casida and Durkin, 2013). According to mammalian studies, the sodium channel is important in the nerve cells and other excitable cells, and it is composed of one pore-forming α-subunit of approximately 260 kDa and four much smaller extra β-subunits of approximately 30-40 kDa. Four homologous repeats compose the α-subunit. Each repeat is composed of six transmembrane segments (S1-S6). The S1–S4 segments in the voltage-sensing. A re-entrant loop (called the P-region) connects the S5 and S6 segments, which serve a pore-forming domain. A small intracellular link between the S4 and S5 segments connect the voltage-sensing domain and the pore-forming domain. The mechanism for the opening is that the S4 segments moves outward to start the voltage-dependent activation, thereby causing the opening of the activation gate, and before the sodium channel is inactivated, the sodium channel may stay open for a few milliseconds. The closing of the activation gate is due to the S4 voltage sensor moving backward, which is called channel deactivation (Dong, 2007; Rinkevich et al., 2013).

As for acetylcholinesterase (AchE), organophosphates and carbamates exert their toxicity via inhibition of the acetylcholinesterase, which is in charge of the hydrolysis of acetylcholine at the synaptic regions of the cholinergic nerve endings (Casida and Durkin, 2013). Acetylcholinesterase is a kind of virtually ubiquitous enzyme that has been isolated or found in a wide range of animals, including mammals, fish, reptiles, insects and other vertebrates or invertebrates. AchE contains two subsites in the active site: an anionic subsite, which includes the positive quaternary amine of acetylcholine as well as other cationic substrates and inhibitors, and an esteratic subsite, which can hydrolyze acetylcholine to acetate and choline. The responsibility of AChE is to rapidly hydrolyze the neurotransmitter Ach, "one of a number of physiologically important neurotransmitting agents and involved in the transmission of nerve impulses to effector cells at cholinergic, synaptic, and neuromuscular junctions" into the inactive products choline and acetic acid (Aldridge and Davison, 1952).

GABA (γ-Aminobutyric acid) receptor is the target site for cyclodiene and phenylpyrazoles, and the GABA receptor can receive the GABA inhibitory neurotransmitter (Cole et al., 1993). In insects, the GABA receptor is a gated chloride-ion channel, a common inhibitory neurotransmission channel in the insect's central nervous system and in the neuromuscular junction (Bermudez et al., 1991). GABA, which serves as the agonist for opening the chloride channel, is the fundamental inhibitory neurotransmitter of insects and mammals (Casida and Durkin, 2013). GABA receptors have several subtypes in insects (Casida and Durkin, 2013; Gilbert and Gill, 2010).

1.4.3.2 Detoxified enzyme

Detoxified enzymes are another powerful weapon that allows insects to resist insecticide. These enzymes consist of two phases of enzymes. The Phase I enzyme is headed by P450s and esterase and can change the non-water soluble insecticide to water-soluble by introducing the polar active group, also called a modification enzyme. Phase II enzyme headed by GST (Glutathione S-transferase), can transfer various endogenous compounds and bind to the product after modification to form a larger and more water-soluble compound, also known as a conjugation enzyme. After the phase I and phase II procedures, insecticide compounds will change to a water-soluble and nontoxic compound and is ready for excretion or storage (Liu et al., 2015).

The first enzyme, cytochrome P450 monooxygenases, represents a diverse class of enzymes found in all insect tissues. P450s are not only involved in important stages for insect growth and development through biosynthetic pathways of ecdysteroid and juvenile hormones, they also have the ability to detoxify insecticides by enhancing its power to metabolize xenobiotics.

The overall reaction of P450 monooxygenase-mediated metabolism can be expressed as followed:

$$RH + O_2 + NADPH + H^+ \longrightarrow ROH + H_2O + NADP^+$$

P450-Fe(III), by which the heme protein is oxidized, binds to the substrate (RH); afterward, the P450 reductase changes from an oxidative state to a reductive state and gives a single electron to the P450-Fe(III)-RH complex, changing the Fe(III) to Fe(II). P450-Fe(II)-RH then binds to an oxygen to form the complex of P450-Fe(II)-RH and O_2. The following is the second electron reduction step: $Cytb_5$ changes from an oxidative state to a reductive state and gives the electron to the P450-Fe(II)-RH and O_2 complex, changing the O_2 to O_2^-. Then, $2H^+$ binds to the O_2^- and splits the complex to H_2O and P-450-Fe(II)-ROH. Finally, the RH changes to the ROH, which is water soluble. P-450-Fe(II) will be used for the next reaction loop (Atkins et al., 1993; Bergé et al., 1998; Feyereisen, 1999; Hemingway and Ranson, 2000). Another study also indicated that the overproduction of cytochrome P450 monooxygenases is an important insecticide mechanism for larvae (Kasai et al., 1998); Moving to the next enzyme, glutathione S-transferases (GSTs) form a large family of multifunctional enzymes related to the detoxification of a myriad of xenobiotic-containing insecticides. One molecule of reduced GSH and one molecule of a second substrate are combined to form a thioester in a GST-catalyzed conjugation reaction (Atkins et al., 1993; Enayati et al., 2005). Two subunits, each approximately 24-28 kDa in size, comprise mostly GSTs. GSTs are cytosolic dimeric proteins and contain two sites for each subunit; these sites include the G site, a highly conserved site that can bind to the tripeptide glutathione and largely consists of amino acid residues found in the N terminal or protein, and the H site is more multifarious in structure and largely formed from residues in the C-terminal (Mannervik, 1985). The final enzyme, esterase, consists of a variety of enzymes and is located in most organisms. Esterase gene amplification and/or occasional overexpression raises the production of the detoxifying proteins (Raymond et al., 1998). Two common esterase loci, estα, and estβ, contribute either alone or together in this type of resistance (Vaughan et al., 1997). The esterase can exert an ability to resist the toxicity of insecticide through rapid-binding and slow turnover of insecticide (Hemingway, 2000).

1.4.3.3 Other mechanisms of insecticide resistance

Changing behavior and reducing penetration has also been proved related to insecticide resistance (Rivero et al., 2010)

Insects can change their behavior to avoid the toxicants of insecticides. One strategy is stimulus-dependent avoidance, which refers to the ability of the insect to detect the toxic substance and calculate the irritant or repellant property of the toxicant to establish an avoidance response (Georghiou, 1972), and another strategy is stimulus-independent avoidance, where an insect can avoid exposure to the toxicant through their innate behavioral traits (Guedes et al., 2009).

Reducing penetration is also another powerful weapon used by insects to fight against insecticides because it can delay the insecticide molecules reaching their target and allow the detoxifying enzymes more time to multiply their effect, thereby resulting in stronger resistance phenotypes (Balabanidou et al., 2018). Polysaccharide, chitin, proteins, and lipids form two primary layers of the insect cuticle. The inner layer, which is the procuticle, contains the chitin and can be divided into the exocuticle (upper and harder part) and the endocuticle (lower and softer part). The outer layer, the epicuticle, is commonly covered by a film of wax and cement without chitin (Balabanidou et al., 2018; Bass and Jones, 2016). Similar to behavior resistance, the cuticle changes underlying insecticide resistance are involved in two main parameters: one is the thickness of the cuticle, which enhances the thickness of all the chitin layers with an overexpressing CP protein or synthesizes the extra hydrocarbons in the abdominal oenocytes by overexpressing the cytochrome P450s (Balabanidou et al., 2016; Huang et al., 2018). Another is the composition changes of the cuticle, which alter the composition of all cuticle through the overexpression of laccase2 to harden the cuticle or overexpress ABC transporters to facilitate the export of cuticular components towards the cuticle (Niu et al., 2008; Pan et al., 2009; Pignatelli et al., 2018).

1.4.4 Molecular basis of insecticide resistance

When scientists try to unlock the secret of the insecticide resistance, genes emerge as being responsible. First, some gene mutations are involved in the target site, which can change the features of the target and make it insensitive; this occurs with the G119S mutation in acetylcholinesterase for organophosphate and carbamate, V1016G, F1534C and V1016I in the sodium channel for pyrethroid and DDT. Consideration is necessary therefore of the insensitivity of the target site, and the way some overexpressed genes show their vital position in resistance: CYP6 and CYP9 subfamilies for P450, alpha-esterase CCEAE3A and CCEAE6A for esterase, GSTE2 and GSTE7 for GST (Faucon et al., 2015; Moyes et al., 2017). Taken together, mutations in the target site (qualitative change) and gene overexpression of the detoxifying enzyme (quantitative change) are the two weapons that allow the insects to protect themselves from the insecticides at the molecular level (Liu et al., 2015).

1.4.4.1 Qualitative change

One or a few genes changing in some specific site of the genome will lead to a significant difference in the ability for insecticide resistance to continue; these changes deal main with the insensitivity of the target. First one, GABA receptor, A302S, the first gene mutation to be reported in the GABA receptor in *Drosophila* (Rocheleau et al., 1993), located in the RDl (resistance to dieldrin) GABA-gated ion channels, has been confirmed to provide resistance to fipronil and is widespread in the field populations of several insects (Du et al., 2005; Pittendrigh et al., 1998). Recently, another mutation that has been reported is R300Q in the brown planthopper. Scientists found that this mutation has synergistic and compensatory abilities when combined with A302S (Zhang et al., 2016). Ace1 gene has been found encoded for acetylcholinesterase and been confirmed to be involved in insecticide resistance (Weill et al., 2003). According to a structural model of AchE1, the G119S substitution would establish an obstruction in the space, making it inaccessible to the inhibitors that depend on substrate weaknesses (Alout and Weill, 2008; Weill et al., 2004). The point mutation or substitutions of the gene relevant to insecticide resistance can cause a single nucleotide polymorphism that leads to the modification of the structures and reduces or even eliminates the binding affinity of the insecticides to the target (Rinkevich et al., 2013). Some other mutations, such as I161V, G265A, F330Y, and G368A have also been confirmed to have the ability to increase the resistance level and provide a wide spectrum of resistance in *Drosophila melanogaster* (Menozzi et al., 2004; Mutero et al., 1994). Another mutation, F455W, has been confirmed to resist organophosphate in *Culex tritaeniorhynchus* through baculovirus-insect cell systems (Nabeshima et al., 2004). The most famous target for insecticide resistance, the sodium channel, has been discovered to have various mutations, which include 11 synonymous mutations (A99S, L1014C/F/S/W, S989P, V1010L, I1011M/V, N1013S, V1016G/I, F1534C, W1594R, N1575Y, and D1763Y) and three nonsynonymous mutations (A109S, L982F, and W1573R) (Liu, 2015). Some of the mutations contribute to the resistance by reducing the binding sites for the insecticide and/or by other mechanisms (Dong, 2007).

1.4.4.2 Quantitative change

Most genes that encode enzymes of great concern, such as detoxifying enzymes, can be overexpressed to obtain more enzymes to digest or resist foreign compounds, such as insecticides. Amplified overexpression, which is the gene copy number increasing but the expressed fold remaining the same as normal, and transcribed overexpression, which is the same amount of genes but their level of expression is higher than normal, are two main ways for genes to overproduce enzyme (Prelich, 2012; Tajouri et al., 2003). Additionally, several regulatory factors have been discovered that can regulate the overexpression of the gene (Barr, 1957; Saavedra-Rodriguez et al., 2012). For example, the CncC and Maf can regulate multiple P450 genes (Kalsi and Palli, 2017). First, carboxylesterases, as one of the major detoxifying enzymes, has been proven to be overexpressed against insecticides. CCEae3a and CCEae6a have proven to be overexpressed in *Aedes aegypti* (Poupardin et al., 2014), and MdαE7 and MdβE2 have been reported as being significantly overexpressed in *Musca domestic* (Feng et al., 2018). Two other esterase genes, estα21 and estβ21, the most common genotypes on an amplicon approximately 28 kbp, are repeated up to 80 times in the genomes of the highly resistant *Culex quinquefasciatus* (Paton et al., 2000; Vaughan et al., 1997). The next enzyme, Cytochrome P450, is one of the most important and omnipotent enzymes involved in insecticide resistance and has been extensively studied. CYP6AA7, CYP9J40, CYP9J34, and CYP9M10 are overexpressed in *Culex quinquefasciatus* (Liu et al., 2011). Other high-resistance species of mosquitoes, such as *Aedes aegypti*, also contain overexpressed genes, such as CYP6CB1, CYP9J2, CYP6M11, CYP6F2, CYP9J21, CYP9J23, CYP4H30, among others (Reid et al., 2014). Even in the less-resistant species, *Aedes albopictus*, CYP6N3, CYP6AE1, CYP6P12, CYP9M6 and CYP9J17 still are overexpressed in the resistant part of the population (Ishak et al., 2016). P450 genes, such as CYP4L5, CYP4L11, CYP6AE11, CYP332A, and CYP9A14, can also be overexpressed in other insects, such as in *Helicoverpa armigera* (Brun-Barale et al., 2010). Finally, Glutathione-S-Transferases has received considerable attention in the last 30 years and has been proven to overexpress against insecticides (Che-Mendoza et al., 2009). Five genes can be used as evidence to confirm GST overexpression: PxGSTE1 in the OP-resistant *Plutella xylostella* (Huang et al., 1998), AgGSTE2 in the DDT-resistant *Anopheles gambiae* (Ortelli et al., 2003), DmGSTD1 in the DDT-resistant *Drosophila melanogaster* (Tang and Tu, 1994), and AaGSTD1 and AaGSTE2 in DDT/pyrethroid-resistant *Aedes aegypti* (Grant et al., 1991; Grant and Hammock, 1992).

1.4.5 Cross resistance

Cross resistance, a phenome where a usually toxic substance is tolerated because of exposure to a similarly acting substance, occurs mainly with insecticides with similar mechanisms of action. Since 2010, 78% of countries with malaria have reported pyrethroid resistance in at least one *Anopheles* malaria vector species according to their monitoring data. Among these countries, 80% of them have mosquitoes that have shown resistance to two or more insecticide classes (Mnzava et al., 2015; Organization, 2016a). *Culex quinquefasciatus* has been confirmed to have the power to resist various types of insecticides, including deltamethrin, chlorpyrifos, fipronil, and imidacloprid (Bisset et al., 1997; Liu et al., 2004b; Su and Cheng, 2014). *Culex pipiens* from Northwestern Tunisia has also shown resistance to pyrethroid after temephos selection (Tabbabi et al., 2017). After selection by organophosphate (temephos), *Aedes aegypti* was observed to resist pyrethroid (deltamethrin) and organophosphate (fenthion) (Rodríguez et al., 2002). Additionally, some other instances of resistance have been reported, such as fenitrothion-deltamethrin cross resistance in *Anopheles* (Brogdon and Barber, 1990); pyrethroid-DDT cross resistance in *Aedes aegypti* (Brengues et al., 2003); and spinosad-fipronil cross resistance in *Culex quinquefasciatus* (Su and Cheng, 2014)

The same insecticide target site may be the reason that cross resistance exists in insecticide resistance. For example; pyrethroid and DDT both target the sodium channel, and the kdr mutation in this channel can weaken the sensitivity of the target. So, *Anopheles gambiae* can resist both pyrethroids and DDT. (Brengues et al., 2003; Severson and Behura, 2012). CYP6Z1 has also been confirmed to play an important role in carbamate-pyrethroid cross resistance (Ibrahim et al., 2016)

1.5 Technology

Technology is the most important aspect advancing insecticide research. From bioassays, scientists can determine which concentration and which class of insecticides is the best to combat insects and how much greater the resistance fold of resistant strains is than that of the susceptible strains (Hoskins and Craig, 1962). From molecular technology, scientists can uncover the secret of insecticide resistance more precisely at the genomic level, such as by observing which gene is overexpressed according to qPCR or by confirming which gene mutation can influence the insecticide resistance through functional study. From gene editing technology, scientists can establish their own mosquitoes with a knock in or knock out genes and observe the differences between these mosquitoes and the wildtype strains. Currently, gene-driven research is providing an opportunity for people to combat the insect without insecticide.

Since the sequencing of the whole genome of mosquitoes (Arensburger et al., 2010; Holt et al., 2002; Nene et al., 2007), scientists have been able to obtain much information from analyzing the data. For example, *Aedes aegypti* has 166 P450 gene sequences and 49 esterase genes (Strode et al., 2008); however, *Culex quinquefasciatus* has 204 P450 gene sequences and 71 esterase genes (Yang and Liu, 2011). According to these data and the result from genome-wide high-throughput sequencing, scientists can obtain a better understanding of the overexpression of resistance genes and the potential mechanisms involved in insecticide resistance, allowing them to select candidate genes for functional studies (Liu, 2015). *In silico* 3-D homology modeling and molecular docking of metabolic enzyme-substrates are efficient tools, are able to provide reasonable explanations for substrate specificities, and can predict the enzyme activities against insecticides (Liu, 2015; Schuler and Berenbaum, 2013). The *Drosophila* transgenic technique is an innovative approach for investigating the specific gene function in insecticide resistance (Coetzee and Koekemoer, 2013). In addition, dsRNA-mediated interference (RNAi), as a gene-silencing technique, can knock down or downregulate the expression of corresponding genes to investigate the role of the specific gene in insecticide resistance (Liu, 2015).

1.6 Reference

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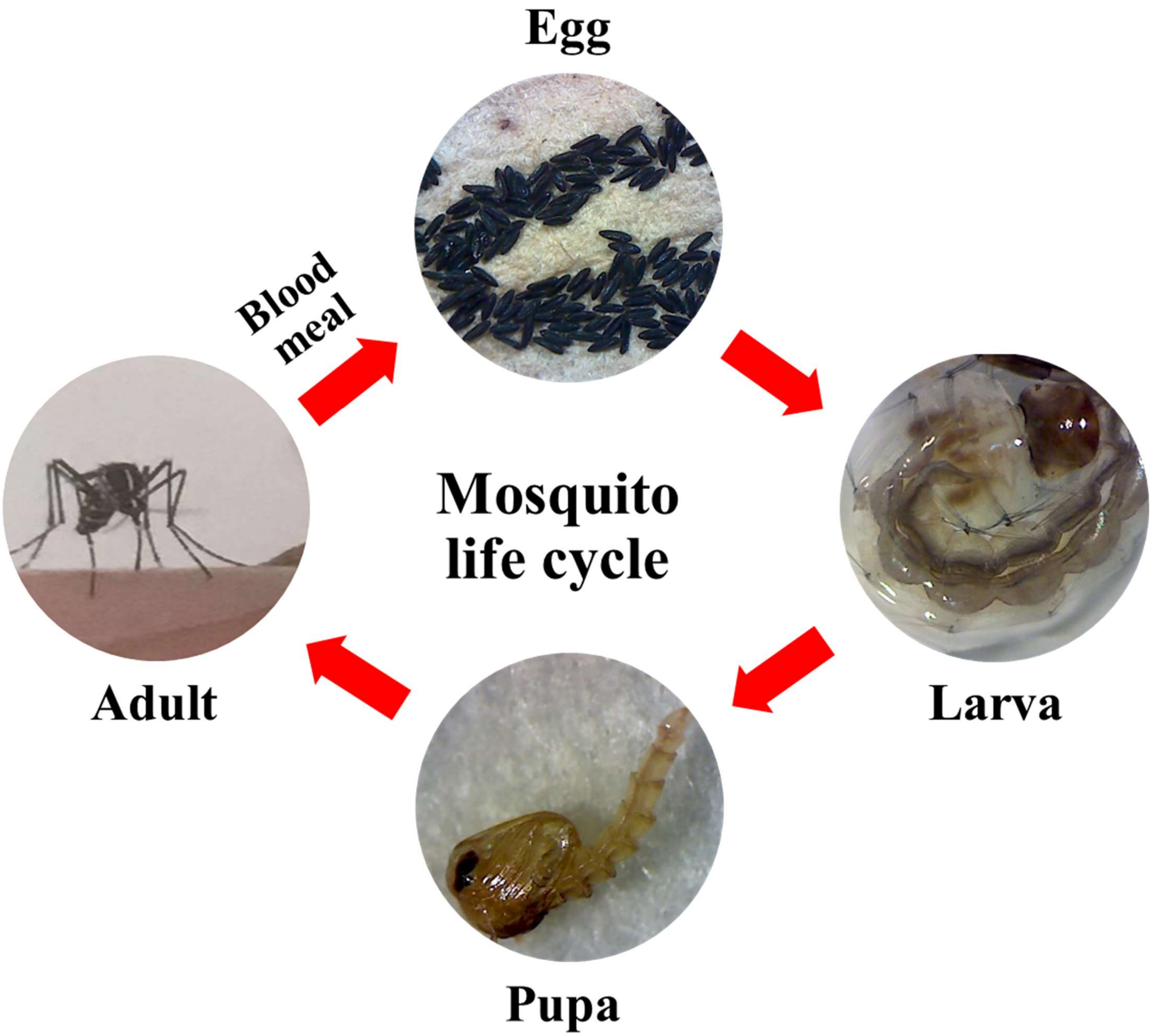

Figure 1: Life cycle of the mosquito

Chapter 2 Research Goals and Specific Objectives

2.1 Goal of the research and objectives.

The goal of this research was to monitor the dynamic changes of the mosquito populations of Alabama and help government officers constitute the best strategy for mosquito population control and mosquito-borne disease prevention. The long-term goal of my research is to uncover the mechanisms of insecticide resistance in mosquitoes. To achieve my long-term goal, two objectives have been achieved: 1) monitoring the dynamic population changes and species diversity of mosquitoes in Alabama from 2017 to 2018 and 2) investigating the resistance level of *Aedes albopictus* in Alabama.

2.1.1 Objective 1: Monitoring the populations and species of mosquitoes in Alabama from 2017 to 2018

During my research, we placed several traps in different locations in Alabama (Tuskegee, Montgomery, Tuscaloosa, Birmingham, Dothan, and Mobile) and collected the eggs from the traps once or twice a month from July 2017 to July 2018. Eggs and larvae were hatched and reared at 25±2°C with an L:D cycle of 12:12 hours (Liu et al., 2004). Adults were supplied with 10% sucrose solution and identified based on the “Keys to the Adult Females and Fourth Instar Larvae of Mosquitoes of Florida (*Diptera, Culicidae*)” (Darsie and Morris, 2003).

To further predict the population changes of mosquitoes in Alabama, the generalized additive model was applied to investigate the relationship between the mosquito populations and the temperature, rainfall, and humidity. I hypothesized that the populations would increase with the temperature.

2.1.2 Objective 2: Investigating the resistance level of *Aedes albopictus* in Alabama.

The ability of *Aedes albopictus* to resist insecticide has been proven in various locations in the world. High LC50 to DDT of the larvae has been found in China (Hunan) (Vontas et al., 2012), high resistance to DDT in adults has been found in Sri Lanka (Ranson et al., 2010), and high resistance to temephos has been observed in adults in Malaysia (Chan et al., 2011). To figure out the resistance status of *Aedes albopictus* in Alabama, adult bioassays were performed to test the resistance of 8 insecticides (deltamethrin, chlorpyrifos, etofenprox, malathion, permethrin, fenitrothion, β-cyfluthrin, and resmethrin). The resistance level was determined by comparing the actual 100% mortality time and diagnostic time provided by the CDC. Larval bioassay was performed to figure out the resistance level for two insecticides (permethrin and fenitrothion).

Probit analysis was performed to calculate the LC50, LC90, and slope. I hypothesized that the *Aedes albopictus* collected in Alabama has not established resistance to insecticides.

2.2 Significance of this study

Insecticide resistance is the largest barrier for scientists as they attempt to develop an effective strategy for combatting mosquito-borne disease. Supervision of the dynamic changes in mosquito populations and monitoring the resistance status of different insecticides is a direct and efficient way to ameliorate the strategy to apply insecticide properly. This research will set a solid foundation of dynamic mosquito population changes and supervision of insecticide resistance levels.

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Chapter 3: Monitoring the Population and Species of Mosquitoes in Alabama from 2017 to 2018

3.1 Abstract

Mosquito population surveillance is fundamental for controlling mosquito-borne disease and can be used to develop more efficient models for predicting population changes (Beck-Johnson et al., 2013; Shaman et al., 2006) and to provide more information to government entities seeking to optimize strategies for combatting disease transmission by mosquitoes. Traps used for collecting mosquito eggs were placed in several locations in Alabama and checked every two or three weeks from July 2017 to July 2018. After counting and identifying the mosquitoes, only 3 species were identified in these collections from Alabama: *Aedes albopictus*, *Aedes triseriatus*, and *Aedes japonicus*. Mosquito populations began to emerge in April and increased quickly in May until reaching a peak in August. Mosquito populations started to develop at 57°F. The suitable range of temperature for mosquito is 75°F to 85°F. According to the GAM (Generalized Additive Model) between environmental parameters and the total population, Mobile and Tuscaloosa are the two cities most suitable cities for mosquitoes, followed by Tuskegee, Montgomery, and Birmingham. Dothan is the least suitable city, with fewer mosquitoes.

3.2 Introduction

Mosquitoes are one of the most widely distributed insects, occurring on every land mass except for Antarctica and a few islands (Mullen and Durden, 2009). Mosquitoes harass humans with behaviors that extend from making noise to transmitting serious diseases during their blood-feeding behavior (Harbach, 2007). As one of the major species in Alabama, *Aedes albopictus* can transfer more than 26 viruses based on experiments including some serious viruses, such as dengue virus, Zika virus, and chikungunya virus (Paupy et al., 2009). *Aedes albopictus* is responsible for the outbreaks of diseases, such as the outbreak of chikungunya in 2005-2007 in all of the Indian Ocean islands and surrounding countries, and that in August 2007 in Italy (Angelini et al., 2008; Paupy et al., 2010). *Aedes albopictus* was first recognized in the United States in 1985 in Texas, and since that time, it has become established in 866 counties of 26 states (Bonizzoni et al., 2013; Lambrechts et al., 2010). *Aedes triseriatus* (Eastern tree hole mosquito) is another common species found in the United States, distributed from Florida to Idaho, and into Utah, and Texas (Farajollahi and Price, 2013). Additionally, it is the major vector of La Cross encephalitis virus, which causes approximately 80 to 100 cases in the United States annually, and it can also spread dengue virus, Eastern equine encephalitis, Western equine encephalitis, West Nile virus, among others. (Freier and Grimstad, 1983; Platt et al., 2007; Styer et al., 2007; Thompson et al., 1972; Unlu et al., 2010; Watts et al., 1974). *Aedes japonicus,* as an invasive species (Schaffner et al., 2009), was first reported in 1998 in North America (Peyton et al., 1999), and since then, it has been recorded in 22 states of the United States (Williges et al., 2008) and in Europe, China and other places. *Aedes japonicus* has been reported to carry West Nile Virus (Turell et al., 2001). Temperature, rainfall, and humidity are three major components of weather, closely linked with our lives, and can influence the dynamic population of mosquitoes. Mosquitoes can increase through shortened periods of development in all stages of the life cycle as the temperature increases (Yang et al., 2009); however, temperatures that are too high (above 95°F) can reduce the survival rate of *Aedes* (Hii et al., 2012).

Several methods have been applied to investigate mosquito populations. Of these methods, larval surveys require relatively small sample sizes but need more time for collection, and pupae surveys can give the absolute measure of population density but require a large sample size. Both larval and pupae surveys are difficult to use when targeting large areas because the mosquito habitats vary from place to place. As for adult surveys, landing-biting counts are the primary method used to investigate the density of mosquitoes. However, this method is not recommended by the CDC because it can increase the possibility of disease for researchers, especially in some areas with ongoing arbovirus transmission (Casas Martínez et al., 2013). Collecting resting mosquitoes with several aspirator devices is the most direct method and can provide the ratio of unfed to fed mosquitoes. BG-Sentinel traps can attract more adult mosquitoes with a combination of visual and olfactory baits, such as light and CO2. Ovitraps, which can attract the gravid female mosquitoes for egg laying are easy and inexpensive and can provide population and epidemiologic information (Service, 1992).

Surveillance of mosquito population dynamics is the principal work required to prevent mosquito-borne diseases and potential increases in mosquito population (Beck-Johnson et al., 2013). In this study, ovitraps have been used to collect mosquito eggs in six cities distributed across Alabama (Mobile, Dothan, Montgomery, Tuskegee, Tuscaloosa, and Birmingham) from 2017 to 2018. Eggs were hatched and reared in lab conditions until the adult stage, when the mosquitoes could be identified and counted.

3.3 Material and methods

3.3.1 *Aedes* mosquito collection

3.3.1.1 Infusion

The infusion was developed using fallen, nearly decomposed, dry leaves and grass, which were visually confirmed. Leaves and grass were collected from the ground near Auburn University. In total, 20 g of leaves and grass and 7 g of yeast (Fleischmann's® Rapid Rise) were added to 4 gallons of water. The mixture was fermented in a black, sealed, plastic bucket at an outside location at a temperature between 20°C and 30°C for approximately 7 days (Obenauer et al., 2009).

3.3.1.2 Site collection

Ovitraps were set from July 2017 to July 2018 in Alabama. Locations were chosen near a river or pool, usually in brush or under trees, which can obstruct rainfall and prevent evaporation. Locations are listed herein (**Table 2 and Figure 2**). Each location contained 3-4 lidless containers (12 cm in height and 11.5 cm in diameter) placed in one basin with a small hole (1 cm diameter) to prevent flooding. Containers were filled with 0.7 l of the infusion and a U-shaped gap was made the top edge of the container sides to prevent the rainwater from submerging the paper completely. Papers were attached to the inside walls of the containers.

3.3.2 Rearing mosquitoes

Mosquitoes were reared at 25±2°C, with a L:D cycle of 12 hours, and they were supplied with a 10% sucrose solution (Liu et al., 2004)

3.3.3 *Aedes spp*. Identification

All identifications were based on the book "Keys to the Adult Females and Fourth Instar Larvae of the Mosquitoes of Florida (*Diptera, Culicidae*)."

In *Aedes spp*.: proboscis is slightly curved (**Figure 3A**); maxillary palpus are much shorter than proboscis; wing scales are long and narrow (**Figure 3B**); pale transverse bands or lateral patches basal on abdominal terga (**Figure 3C**).

In *Aedes albopictus*, *Aedes japonicus* and *Aedes triseriatus*, the proboscis lacks definite pale-scaled band near middle (**Figure 3A**), except for *Aedes triseriatus* which does not have the pattern on the scutum **(Figure 4A)** and basal white on the hindleg, but tooth less than 0.3 of claw (**Figure 4B**). The remaining two species are similar: *Aedes japonicus* has lyre-shaped pattern on the scutum (**Figure 5A**), their 4, 5 hind tarsomeres are dark-scaled (**Figure 5B**). *Aedes albopictus* has the basal white band on the 4 hind tarsomeres, and entirely white on 5 hind tarsomeres (**Figure 6A**). However, *Aedes albopictus* only have a single narrow strip of white scales on the scutum (**Figure 6B**).

3.3.4 Weather data

The data for high temperature, average temperature, low temperature, total rainfall and humidity for each month were downloaded from the underground weather website: https://www.wunderground.com/.

3.3.5 Analysis

Population, percentage, and average data were calculated with Excel. The GAM (Generalized additive model) of the environment parameter to total population; each environment parameter to the total population; high-temperature, min-temperature, and average-temperature to the total population; and environment parameter to each species was conducted by R 3.5.1 base on the negative binomial distribution and log function in the mgcv package.

3.4 Result

3.4.1 Population dynamics changes from 2017-2018 in Alabama

A total of 16,749 mosquitoes were collected in 15 locations near six cities during this research: Tuskegee, Tuscaloosa, Birmingham, Montgomery, Mobile and Dothan (**Table 2**). Three species were identified among the population: *Aedes albopictus*, *Aedes japonicus* and *Aedes triseriatus* (**Figure 7G**). *Aedes albopictus* is the primary species in Alabama, accounting for more than 80% of the total mosquito population from the survey, and 13,419 were collected in total. *Aedes albopictus* followed, accounting for 10% of the total population, and 1,773 were gathered in total. *Aedes japonicus*, as the last one, also accounted for approximately 10% of the total population, and 1,602 were obtained in total (**Table 3**).

Among the six cities, Mobile and Tuscaloosa were the two cities with the most abundant mosquitoes, followed by Tuskegee, Montgomery, and Birmingham. Dothan is the safest city with the smallest abundance of mosquitoes

(**Figure 7H**). Mobile was the only city with *Aedes albopictus* only (**Figure 7C**), and Montgomery only had *Aedes albopictus* and *Aedes triseriatus* (**Figure 7B**); The remaining cities-Tuskegee (**Figure 7E**), Tuscaloosa (**Figure 7A**), Birmingham (**Figure 7D**), and Dothan (**Figure 7F**) had all 3 of the species we identified: *Aedes albopictus*, *Aedes japonicus* and *Aedes triseriatus* (**Table 3**).

We set up traps at Tuskegee, Tuscaloosa, Montgomery, and Birmingham from July 2017 to October 2017 and collected from March 2018 until now. During this period, August was the month with the most abundant mosquitoes in Tuskegee, Montgomery, and Tuscaloosa, whereas Birmingham had the largest population of mosquitoes in September 2017. We did not collect any eggs in March, which is the month with the lowest temperature and relative humidity (**Figure 8 B, C**). Mosquito populations began to increase along with temperature and relative humidity in April and May (**Figure 8 B, C**). However, the dynamic changes in the mosquito population do not follow the rainfall changes (**Figure 8A**). *Aedes japonicus* was the pioneer species, emerging first in April at Tuskegee and Birmingham. By contrast, *Aedes albopictus* was the primary species, emerged first and was the most abundant species at all time points in Montgomery and Tuscaloosa (**Figure 9**).

3.4.2 Generalized additive model to investigate the relationship between the population and weather

The generalized additive model was applied to determine the potential relationship between the population and rainfall, humidity, min-temperature, high-temperature, and average-temperature. I consider min-temperature, high-temperature, and average-temperature as one parameter because they are all related to the temperature. So, the three parameters used in the generalized additive model were humidity, rainfall, and temperature. According to the model (AIC value: 403.3559), the rainfall (p-value: 0.0658), humidity (P-value: 0.0258) and temperature (p-value: $5.73e^{-08}$) can explain 79.4% of the dynamic changes of the mosquito populations.

According to the relationship between average-temperature and the population based on the model, no mosquitoes were present until the average-temperature reached approximately 57°F. The suitable temperature range is 75°F to 85°F; however, the higher temperature can result in a decrease in mosquitoes (**Figure 10**). The relationship between population and humidity is quite different than the temperature based on the same model. Mosquitoes start to emerge at approximately 67% relative humidity. When the relative humidity exceeded 80%, the mosquito population is significantly larger than when the relative humidity is below 70% (**Figure 11**).

We wanted to determine which parameter could influence the population dramatically. Therefore, we constructed the model between the population and three parameters separately (population ~ humidity, population ~ rainfall, and population ~ temperature). According to the results, temperature is the most influential parameter, which can explain 64.9% of the dynamic population change with the $1.39e^{-13}$ p-value. Temperature was followed by humidity, which can explain 14.4% of the dynamic population change with the 0.00375 p-value. Humidity was followed by rainfall, which can only explain 0.00167% of the dynamic population change, with a 0.98 p-value. We also tried to determine which temperature (min-temperature, high-temperature, and average-temperature) is essential for mosquito population changes. So, we consider the three types of temperature independently and constructed a generalized additive model between population and temperature. However, we were not able to determine which type of temperature was the most efficient way to influence the population of mosquitoes (P-value of average-temperature: 0.298; P-value of min-temperature: 0.610; P-value of high-temperature: 0.754.)

We established a clear relationship between the temperature and *Aedes albopictus* regarding the proportion of the total population (p-value:0.00811) and between temperature and *Aedes triseriatus* regarding the proportion of the total population (p-value: 0.00179). However, we have not determined the relationship between the three parameters and *Aedes japonicus* (P-value of humidity: 0.337; P-value of rainfall: 0.610; P-value of temperature: 0.595). Interestingly, at the range of 75°F to 77°F, the proportion of *Aedes triseriatus* increases to 15%, However, the *Aedes albopictus* is still the primary species even though its proportion drops to 60% (**Figure 12**).

3.5 Discussion

Among the three species we found in Alabama, *Aedes albopictus*, which we found in all the locations trapped, was first reported in 1985 in Texas and has now been established in 866 counties of 26 states (Bonizzoni et al., 2013; Lambrechts et al., 2010). The shipment of used tires from countries with *Aedes albopictus* was regulated to prevent further importation by the Public Health Service Act in 1988 (Moore and Mitchell, 1997). As for the state of Alabama, *Aedes albopictus* was first identified in Mobile in 1987 and then increased quickly and replaced *Aedes aegypti* in 1990 (Alto and Juliano, 2001). *Aedes triseriatus* is a native *Aedes* species, distributed almost throughout the entire United States (Farajollahi et al., 2011). We found colonies of *Aedes triseriatus* in Tuskegee, Tuscaloosa, Birmingham, Dothan, and Montgomery (in 5 of the 6 cities studied) (Denlinger et al., 2012; Mathias et al., 2007);

Aedes japonicus was first reported in 1998 in North America (Peyton et al., 1999); it was found in Tuskegee, Tuscaloosa, Birmingham, and Dothan in this study.

Aedes albopictus has been recognized as the primary species in Alabama in this study. In Florida, *Aedes albopictus* was also the super competitor compared to native *Aedes* species in an earlier study (Lounibos et al., 2001). According to the generalized additive model of average-temperature effects on *Aedes albopictus* and *Aedes triseriatus*, *Aedes albopictus* has the upper hand over *Aedes triseriatus* in the wild. When the temperature reaches a suitable range for mosquitoes, the population of *Aedes triseriatus* can increase to 15% of the total population. However, *Aedes albopictus* is still the primary species. Other research has also confirmed the superiority of *Aedes albopictus* when in competition with other species (Paupy et al., 2009), such as *Aedes japonicus* (Armistead et al., 2008), *Aedes triseriatus* (Brady et al., 2013), and *Aedes aegypti* (Pumpuni et al., 1992; Ruiz et al., 2004), based on the higher immature survivorship in all conditions (Li et al., 2014), the stronger ability to restore energy to resist the lack of food (Barrera, 1996), positive population growth at higher combined density (Smith et al., 2004), more successful survival in the presence of predators and less time needed for hatching in *Aedes albopictus* (Lounibos et al., 2001).

Among the sampled cities, Mobile and Tuscaloosa are the two most favored cities for mosquitoes. Mobile is a highly urbanized city with a huge human population and density and has a large population flow as a seaport city (Carbajo et al., 2006; Li et al., 2014; Ruiz et al., 2004). Tuscaloosa, similar to Mobile, is able to accept a huge number of students per year and provides high-frequency activities (Paupy et al., 2001; Rochlin et al., 2016). A large number of humans and extensive urbanization can contribute to readily available blood meals and more potential breeding sites to attract mosquitoes, especially for *Aedes albopictus* (Obenauer et al., 2017; Russell et al., 2002). In addition, the larger the population of mosquitoes, the higher the risk of the prevalence of mosquito-borne diseases (Smith et al., 2004) After Mobile and Tuscaloosa, Birmingham and Montgomery are the two cities with similar human populations and density, and these cities also show the same ability to attract mosquitoes (Eddleston et al., 2008; Eskenazi et al., 1999; Naqqash et al., 2016; Petrić et al., 2014; Rochlin et al., 2016). Tuskegee has more mosquitoes than Dothan, while also having a smaller human population and a lower density. However, Tuskegee has more forested area compared to Dothan, and can attract more *Aedes* species, such as *Aedes triseriatus* and *Aedes japonicus. Aedes japonicus* and *Aedes triseriatus* live in forested areas and breed in natural water containers, such as tree holes and rock holes. These mosquitoes can easily move to the forest to avoid competition with *Aedes albopictus.*(Barker et al., 2003; Canyon et al., 1999; Leisnham and Juliano, 2012).

We did not collect any eggs in any location until April; according to our GAM-analysis, mosquitoes begin to lay eggs and breed when the average temperature is approximately 55°F. The average temperature of all locations in March is 56°F, which is still too cold for the mosquitoes to reproduce and breed. Previous research shows the minimal threshold of the immature stage of development should be above 50°F (Apperson et al., 2004). The min-temperature of all locations in March was below 50°F (Tuskegee 44°F, Montgomery 45°F, Tuscaloosa 45°F in March and 48°F in April, Birmingham 44°F). The temperature should be the primary factor for mosquitoes not emerging in March (Carbajo et al., 2006). The maximum number of mosquitoes we observed was in August and September, when the average temperature was approximately 79°F-80°F. Compared to our GAM-analysis, the optimal average-temperature was approximately 78°F-79°F, which is similar to the actual temperature. Others also report the suitable temperature of mosquitoes should be in the range of 77°F-86°F (Beck-Johnson et al., 2013), which allows for greater intrinsic rates (Alto and Juliano, 2001) and optimal survival rate (Brady et al., 2013). According to the GAM, the mosquito population starts to decrease when the temperature becomes too high, and other studies have shown that the excessively high temperatures are not conducive to mosquito survival (Hii et al., 2012). Other than temperature, humidity is also an important environmental factor and can influence the population of mosquitoes (Yamana and Eltahir, 2013). According to our Generalized Additive Model of the relationship between the relative humidity and the mosquito population, high relative humidity contributes to a greater mosquito population than low relative humidity because it is helpful for female mosquitoes to lay more eggs and increase the opportunity for mosquito survival (Canyon et al., 1999; Costa et al., 2010)

Diapause, a method used by mosquitoes and other insects to pass the winter and reproduce the next generation in the next spring, is similar to dormancy, which is hormonally programmed in advance of its onset and is not immediately stopped in response to suitable conditions. Not only do the mosquitoes experience this diapause, but the virus inside the body of a mosquito can also use this period to bypass bad conditions (Nasci et al., 2001). The mechanism of diapause is still unknown; however, photoperiod (Pumpuni et al., 1992), temperature, rainfall, relative humidity, the changes of hormone concentration (Denlinger et al., 2012), and specific gene expression (Mathias et al., 2007) can be used to influence the diapause.

The interesting thing is that *Aedes japonicus* is the pioneer species in Tuskegee and Birmingham, whereas *Aedes albopictus* is the pioneer species in Tuscaloosa and Montgomery. *Aedes japonicus* is not the pioneer species in Tuscaloosa and Montgomery, probably because *Aedes albopictus* is present, and nearly controls the two cities, with populations accounting for 95% of the mosquitoes in Montgomery and 88% in Tuscaloosa. Possibly, other species may emerge first but are still in too low a number to be observed. However, *Aedes albopictus* only accounts for 43% of the total population in Tuskegee and 77% in Birmingham. Altogether, *Aedes japonicus* emerges as the pioneer species in spring.

3.6 Reference

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Table 1: Trap ID and Location

Trap ID	Latitude	Longitude	Nearby street
Tuskegee	32.42843	-85.675884	Macon Dr.
Montgomery 17	32.375406	-86.325613	Herron St.
Tuscaloosa 17a	33.210505	-87.576259	Jack Warner Pkwy
Tuscaloosa 17b	33.209917	-87.577334	Jack Warner Pkwy
Birmingham 17	33.40285	-86.665474	Farley Ln
Montgomery 18	32.394465	-86.313837	Abbie St
Tuscaloosa 18	33.215104	-87.565766	21st Ave
Birmingham 18	33.376954	-86.667808	Mountain Top Rd
Mobile 1	30.656094	-88.101883	Halls Mill Rd
Mobile 2	30.698939	-88.053985	Short Bloodgood St
Mobile 3	30.68116	-88.066831	S Ann St
Mobile 4	30.688724	-88.054502	St Francis St
Dothan 1	31.225648	-85.374794	Plant St
Dothan 2	31.111362	-85.594995	N Morris St
Dothan 3	31.233573	-85.440601	Ginnalou Dr
Dothan 4	31.240622	-85.475132	W Main St
Dothan 5	31.228347	-85.368905	Basin Ave
Dothan 6	31.227118	-85.372868	E. Burdeshaw St
Dothan 7	31.187179	-85.599634	N State Hwy 103

Table 2: Total population of mosquitoes collected in Alabama from 2017 to 2018

	Species						
Location	*Aedes albopictus*		*Aedes triseriatus*		*Aedes japonicus*		**Total**
	number	percentage[a]	number	percentage[a]	number	percentage[a]	
Tuskegee	929	43%	132	6%	1,085	51%	2,146
Montgomery	2,311	95%	130	5%	0	0%	2,441
Tuscaloosa	5,611	88%	638	10%	116	2%	6,365
Birmingham	2,027	77%	293	11%	313	12%	2,633
Dothan	1,145	63%	580	32%	88	5%	1,813
Mobile	1,396	100%	0	0%	0	0%	1,396
Total	13,419	80%	1,773	10%	1,602	10%	16,749

a Percentage is calculated by diving the number of each species by total population.

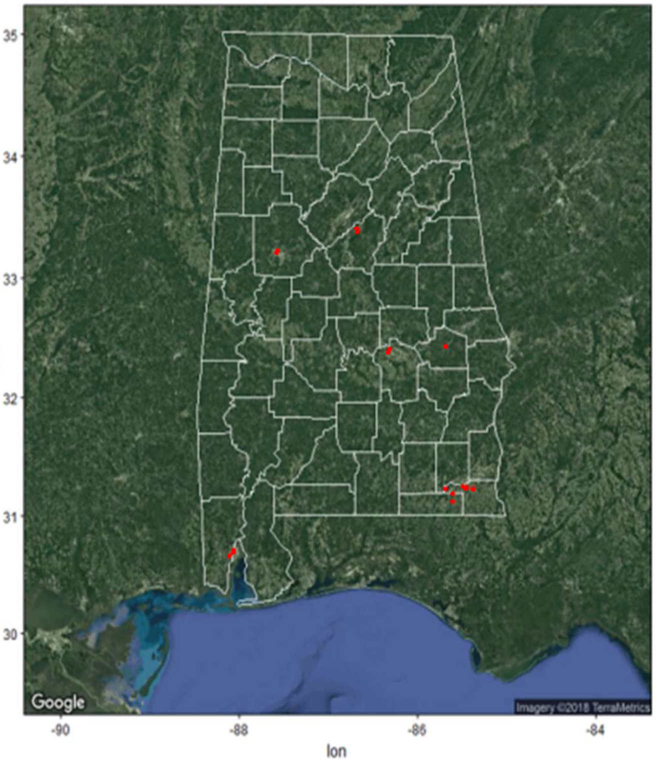


Figure 2: Trap locations in Alabama

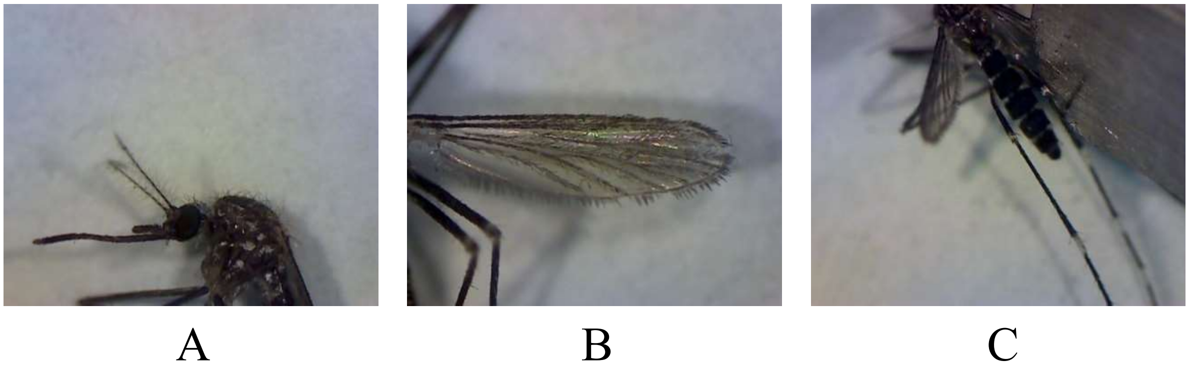


Figure 3:The characteristic of *Aedes spp.* (A) Slightly curved proboscis is much longer than maxillary palpus. (B) Wing scales are long and narrow. (C) Pale transverse bands or lateral patches basal on abdominal terga.

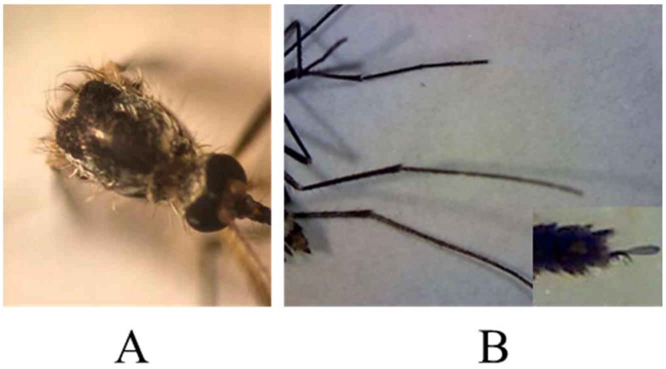


Figure 4: The characteristic of *Aedes triseriatus*. (A) Scutum with median, longitudinal stripe of dark brown scales and silvery white scales laterally. (B) No pale and white bands on the hindlegs, but tooth less than 0.3 of claw.

Figure 5: The characteristic of *Aedes japonicus*. (A) Scutum with lyre-shaped pattern. (B) 4,5 hind tarsomeres are dark-scaled.

Figure 6: Characteristics of *Aedes albopictus*. (A) White bands on four hind tarsomeres and five hind tarsomeres is totally white. (B) Only one single narrow stripe of white scales on the scutum.

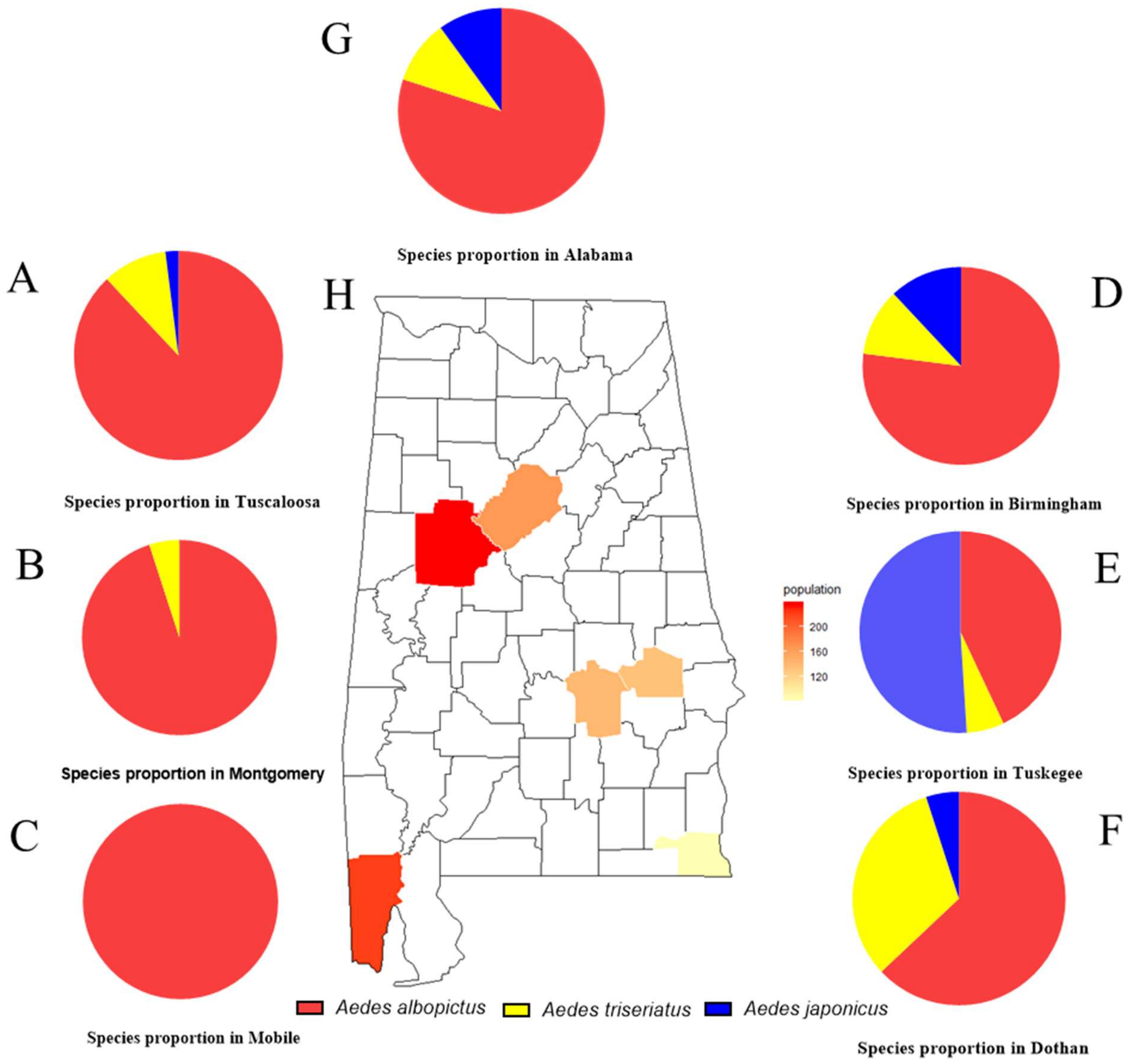


Figure 7: *Aedes* mosquito populations in five countries of Alabama. (A) Species proportion in Tuscaloosa. (B) Species proportion in Montgomery. (C) Species proportion in Mobile. (D) Species proportion in Birmingham. (E) Species proportion in Tuskegee. (F) Species proportion in Dothan. (G) Species proportion in Alabama state. (H) Heatmap of the mosquito population in Tuskegee, Tuscaloosa, Montgomery, Birmingham, Dothan and Mobile.

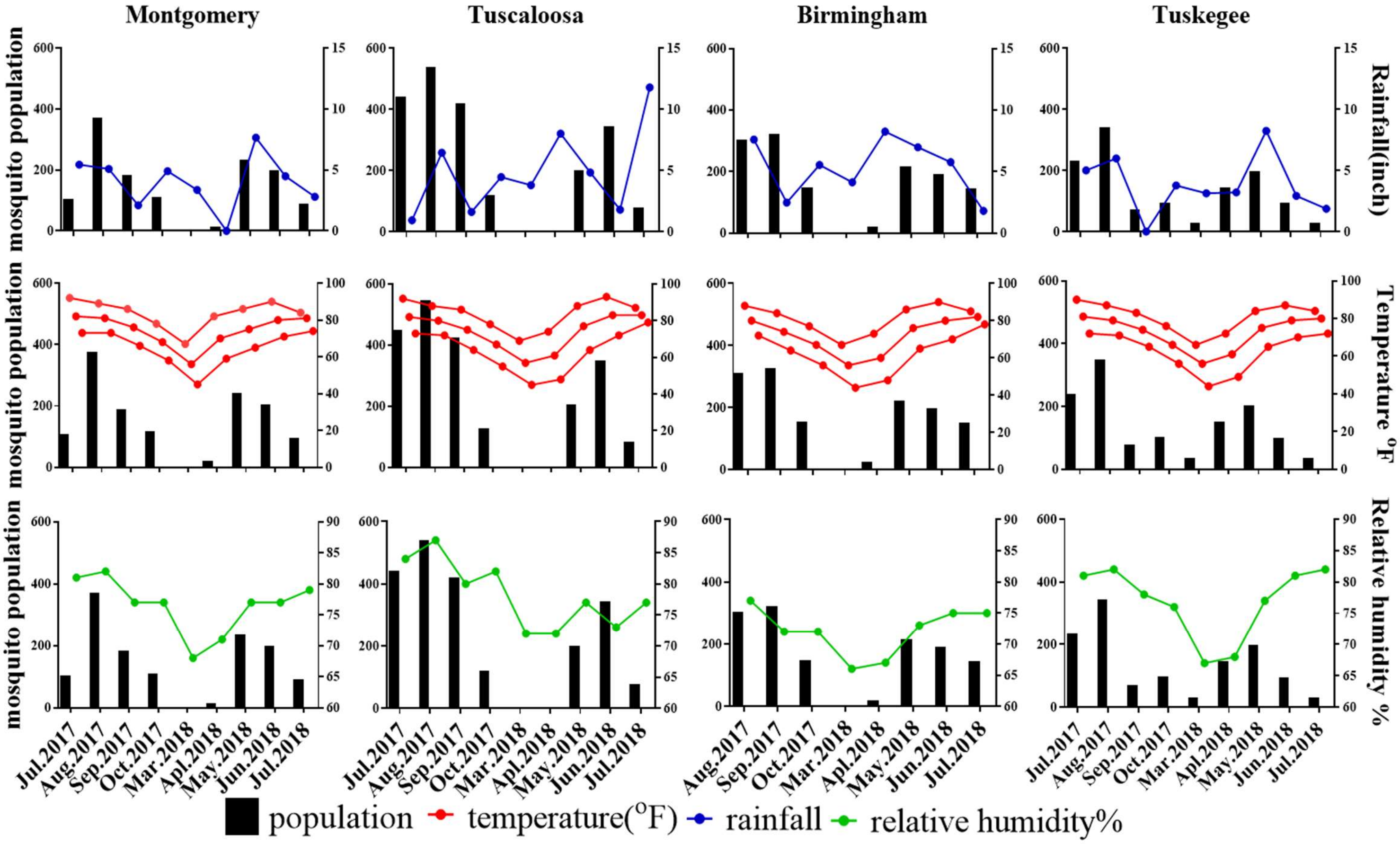
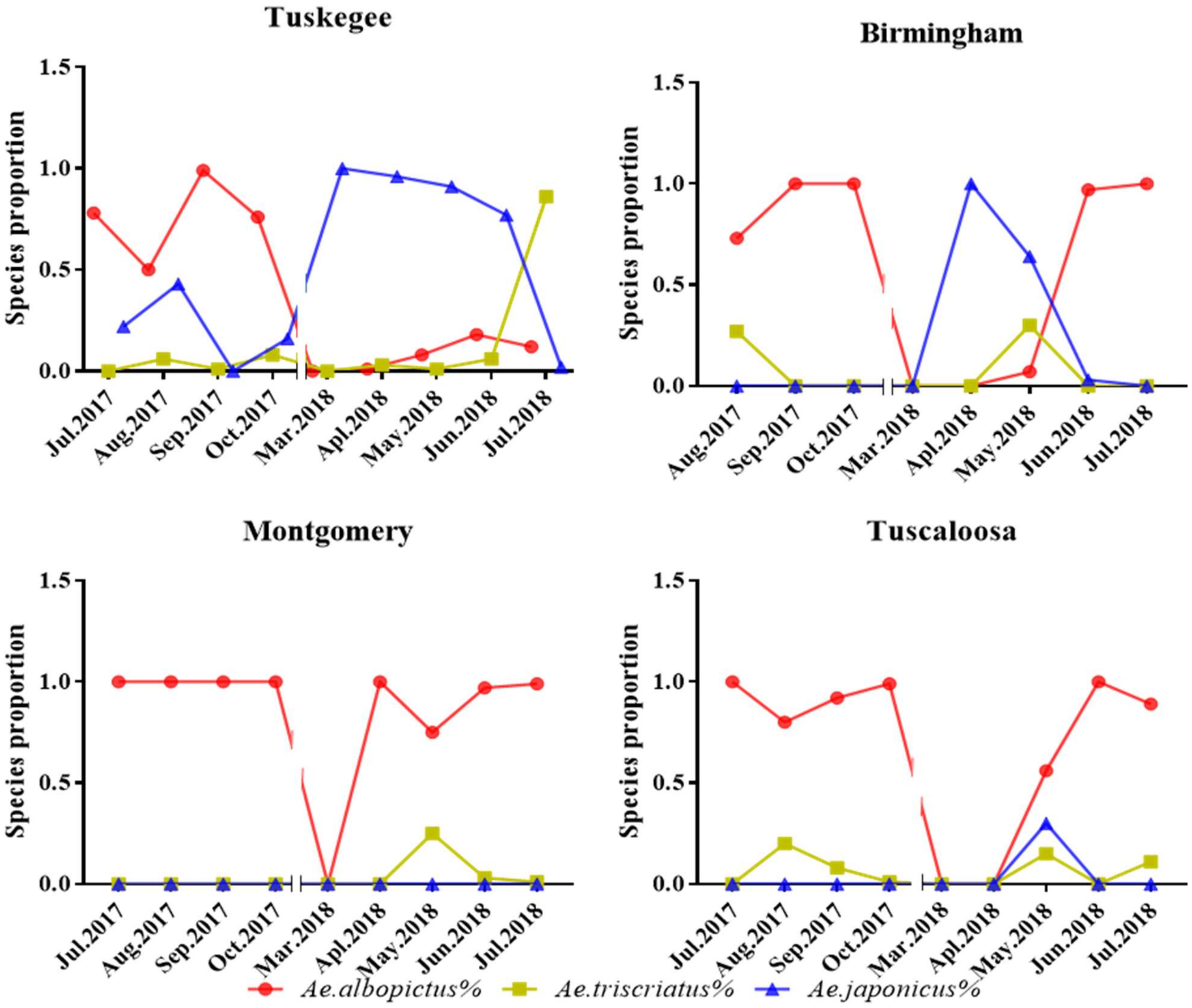


Figure 8: Dynamic mosquito population changes in Tuskegee, Montgomery, Tuscaloosa, and Birmingham from 2017 to 2018. (A) Dynamic mosquito population and rainfall changes. (B) Dynamic mosquito population and temperature changes. (C) Dynamic mosquito population and relative humidity changes.


Tuskegee

Birmingham

Montgomery

Tuscaloosa

Figure 9: Proportion changes of each species in Tuskegee, Montgomery, Tuscaloosa, and Birmingham

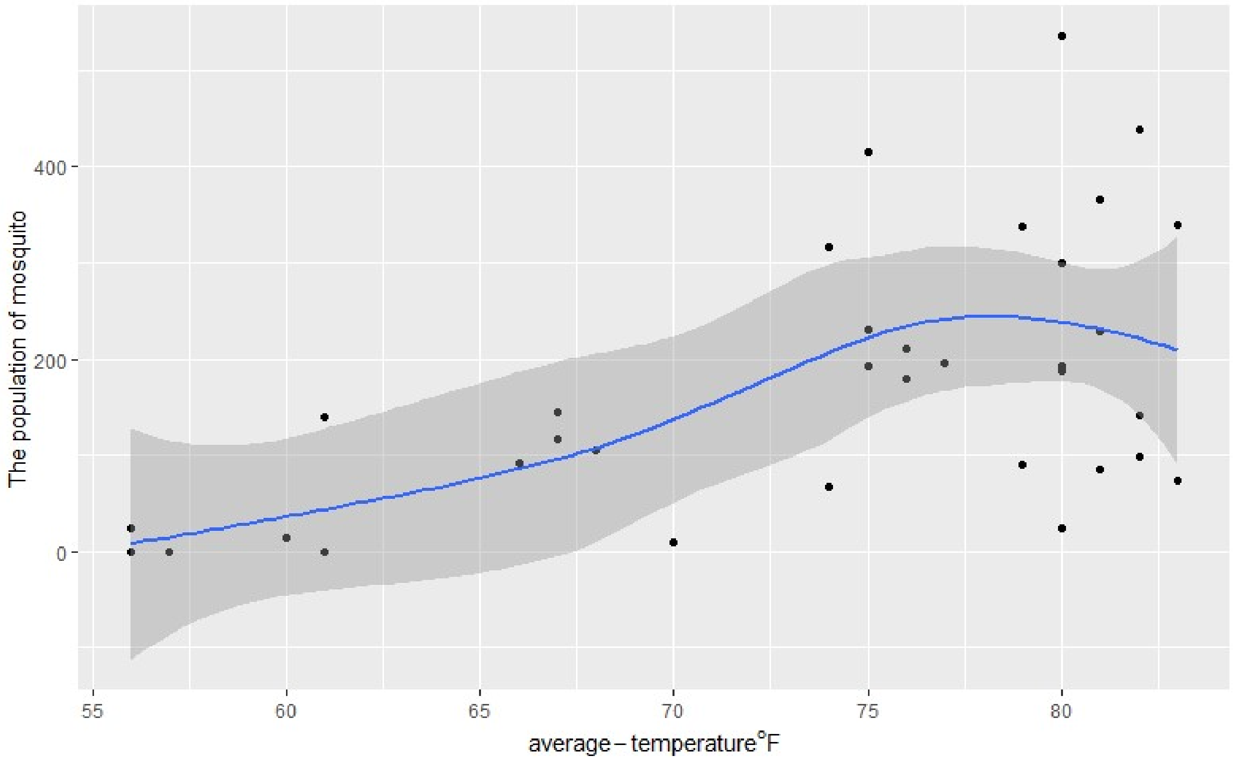


Figure 10: Relationship between average temperature and population changes based on the generalized additive model.

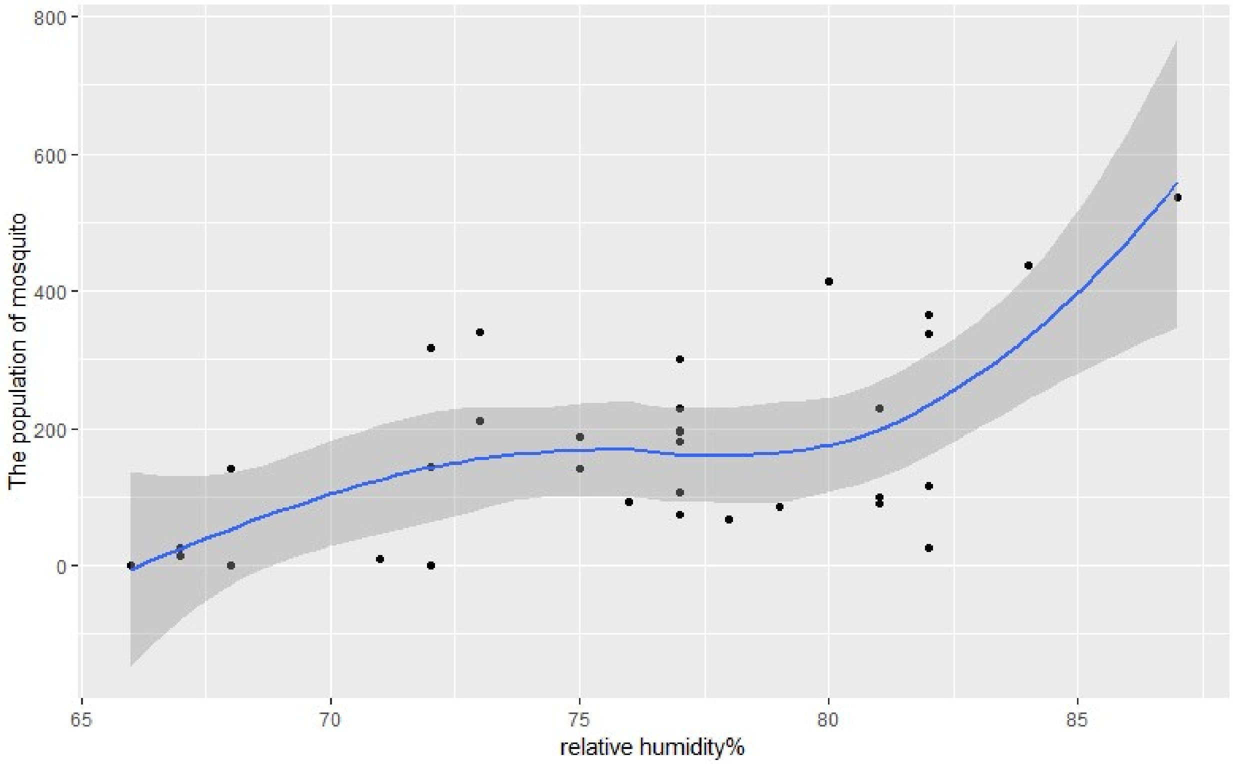
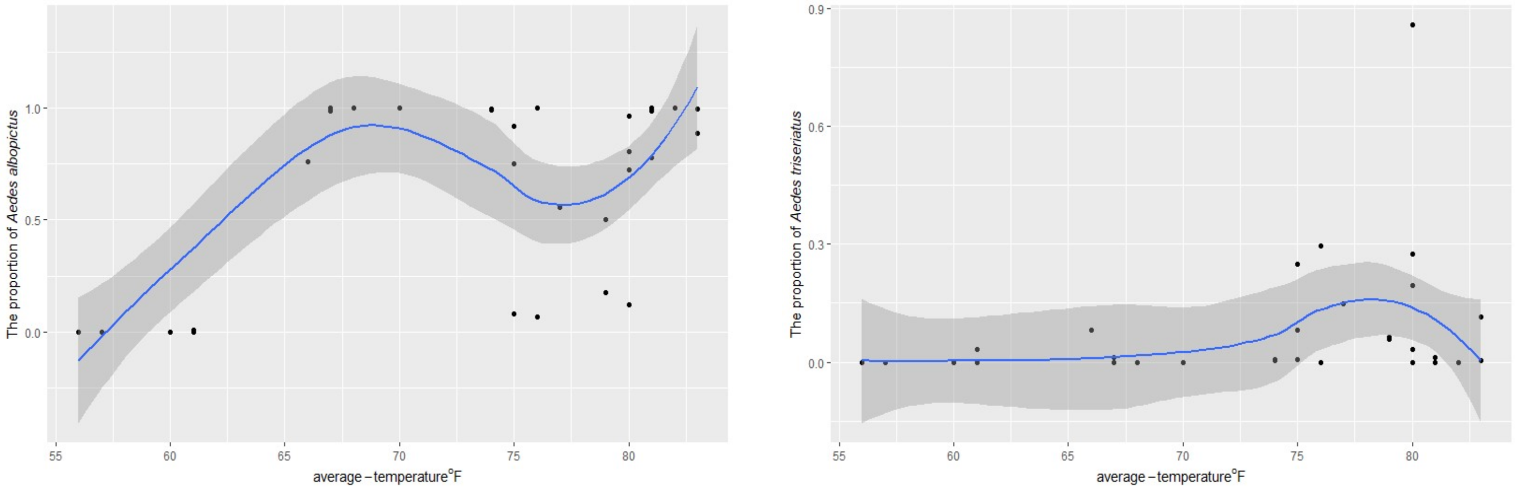


Figure 11: Relationship between humidity and population changes based on the generalized additive model.



A

B

Figure 12: (A) Relationship between the average temperature and proportion of *Aedes triseriatus* based on the generalized additive model. (B) Relationship of the average temperature and proportion of *Aedes albopictus* based on the generalized additive model.

Chapter 4: Investigating the resistance level of *Aedes albopictus* in Alabama

4.1 Abstract

Aedes albopictus, one of the primary vectors of mosquito-borne disease transmission, is a dramatic threat to human health. Among the various methods used to combat mosquitoes, such as setting up traps, spraying chemicals, and cleaning house, the use of insecticides is still the most efficient approach. However, inappropriate and intense use of insecticides leads to insecticide resistance; for example, *Aedes aegypti* has been confirmed to be resistant to various types of insecticides, including temephos, malathion, permethrin, DDT and deltamethrin (Chen et al., 2016; Goindin et al., 2017; Ishak et al., 2015; Kamgang et al., 2017; Marcombe et al., 2014; Seixas et al., 2017). Similarly, *Aedes albopictus* can resist chlorpyrifos, pyrethroid, and carbamate (Li et al., 2018; Marcombe et al., 2014).

In my study, adult and larval bioassays were conducted to test the resistance status of *Aedes albopictus* from Alabama (Huntsville, Mobile, Dothan, Birmingham, Tuskegee, Tuscaloosa, and Montgomery). According to the results, *Aedes albopictus* from all locations showed similar differences between the time to 100% mortality and diagnostic time except for permethrin, fenitrothion, and resmethrin. Mobile has the largest proportion of resistant strains. Furthermore, we suggest that β-cyfluthrin and deltamethrin be used to replace permethrin and malathion.

4.2 Introduction

Mosquito-borne diseases are diseases caused by bacteria, virus, and parasites transmitted by mosquitoes, and these diseases endanger nearly 700 million people and result in more than one million deaths annually (Caraballo and King, 2014). Because of their broad distribution, mosquitoes can transmit these diseases to thousands of countries. *Aedes albopictus* and *Aedes aegypti* are two primary vectors responsible for disease transmission. *Aedes albopictus*, also known as the Asian tiger mosquito, can spread more than 26 viruses including Zika virus, *Flaviviridae* (dengue virus) and *Alphavirus* (chikungunya virus) (Paupy et al., 2009). *Aedes albopictus* is responsible for several outbreaks of disease, serving as the only or the main vector of the 2005-2007 outbreak of chikungunya virus. *Aedes albopictus* was confirmed as the vector for that outbreak during the first European outbreak reported in Italy in August 2007 (Angelini et al., 2008; Paupy et al., 2009; Paupy et al., 2010) and as the primary vector in the Gabonese outbreak, according to the Entomological survey (Paupy et al., 2010). *Aedes albopictus* mainly comes from Asia and from islands in the Indian and Pacific Oceans (Knudsen, 1995). After the 1980s, *Aedes albopictus* had become established in other places

(Benedict et al., 2007): in Albania in 1979 (Adhami and Reiter, 1998), in America in 1985 (Lambrechts et al., 2010), and in Brazil in 1986 (Gratz, 2004). Until now, *Aedes albopictus* has been reported in 26 states in America (Bonizzoni et al., 2013; Lambrechts et al., 2010). *Aedes aegypti*, also known as the yellow fever mosquito, can transmit serious diseases, such as dengue fever, chikungunya, Zika fever and yellow fever (Kraemer et al., 2015). All of these diseases have caused severe damage throughout history. Dengue contributes more than 5 million cases each year (Organization, 2009), and more than 1.5 million cases of chikungunya occurred in 2006 (Pialoux et al., 2007), and yellow Fever infects 200 thousand people per year (Barnett, 2007; Gubler, 2004). *Aedes aegypti* originates in Africa (Brown et al., 2014), was introduced into New York along with the slave trade from Africa to the Americas, and then spread globally to tropical and subtropical regions of the world (Brown et al., 2014).

The most efficient way to control mosquito-borne diseases is to control the population of mosquitoes, decreasing the population to decrease the possibility of disease transmission. Plenty of methods have been applied to regulate the population. For example, environmental sanitation methods such as removing water from cans, tree holes, and flower pots to prevent egg laying can be applied (Baldacchino et al., 2015; Martinez-Ibarra et al., 2002). Biological methods have included the use of fungi, fish, insects or other natural enemies to kill the mosquitoes (Baldacchino et al., 2015; Chandra et al., 2008; Martinez-Ibarra et al., 2002) Mechanical methods utilize traps or bed-nets to prevent mosquito bites, and chemical methods, such as indoor insecticide sprays, have been used. Among these methods, the insecticide is the most broadly used approach against mosquitoes because it can decrease the population rapidly. Organophosphate insecticides and pyrethroid insecticides are two of the most widely applied insecticides, and as much as 262 tons and 39 tons per year, respectively, have been used (Manjarres-Suarez and Olivero-Verbel, 2013; Zaim et al., 2007).

Unfortunately, the intensive and inappropriate use of insecticides can lead to resistance problems (Feng et al., 2018; Liu et al., 2004): *Aedes aegypti* has been confirmed as resistant to a variety of insecticides, including temephos, malathion, deltamethrin, permethrin, DDT, chlorpyrifos and propoxur, worldwide (Chen et al., 2016; Kamgang et al., 2017; Li et al., 2018; Marcombe et al., 2014; Ponlawat et al., 2005); *Aedes albopictus* is less resistant compared to *Aedes aegypti* (Hamdan et al., 2005) but still has been reported as significantly resistant to three major classes of insecticides—chlorpyrifos, pyrethroids, and carbamates—worldwide (Goindin et al., 2017; Ishak et al., 2015; Marcombe et al., 2014; Rodríguez et al., 2007; Seixas et al., 2017)

In this study, we used the *Aedes albopictus* collected from Mobile, Dothan, Montgomery, Tuskegee, Tuscaloosa, and Birmingham to test their resistance level through adult bioassay and larval bioassay.

4.3 Method

4.3.1 *Aedes albopictus*

Aedes albopictus was collected in six cities in Alabama (Mobile, Dothan, Montgomery, Tuskegee, Tuscaloosa, Birmingham) from 2017 to 2018. *Aedes albopictus* is an important pest, has existed in Alabama for more than 20 years, and has been exposed to several insecticides: chlorpyrifos, malathion, permethrin, fenitrothion, etofenprox, deltamethrin, β-cyfluthrin, resmethrin). All mosquitoes were reared at 25±°2C under a photoperiod of 12:12 (L:D) h (Liu et al., 2004).

4.3.2 Insecticides

A series of insecticides were chosen to represent different types of insecticides with different modes of action in this study to measure the toxicity, resistance or cross resistance patterns, and probable mechanisms in resistance in mosquitoes. Chlorpyrifos (99.3%), Fenitrothion (95.8%), Etofenprox (99.1%), and β-cyfluthrin (99.9%) were supplied by Sigma-Aldrich®; Malathion (99.2%) and Resmethrin (98%) were obtained from Chem Service, Inc.; Deltamethrin (99.9%) and Permethrin (94.34%) were provided by FMC Crop (Princeton NJ).

4.3.3 Adult bioassay

Diagnostic time, the accepted time for an insecticide to kill 100% of susceptible mosquitoes, and the Diagnostic dose, the dose of insecticide that can kill 100% of mosquitoes in the accepted time, were used, according to the instructions for the CDC bottle bioassay. Furthermore, the diagnostic doses were diluted according to the size of the bottle we used in our assay relative to the size of the CDC bottle (**Table 6**) (Control and Prevention, 2010; Organization, 2016)

Each dose of 200 µl insecticide at the diagnostic concentration was added to each bottle (20 ml disposable scintillation vials VWR$^{®}$). Bottles were coated by swirling and inverting. After that, the bottles were placed on one side and gently rolled, so the insecticide could cover all the inside of the bottle. The bottle caps were then removed, and the bottles continued rolling until no signs of liquid were visible. Bottles were laid down and covered with paper towel to protect them from light and allowed to rest for approximately 2-3 hours (Control and Prevention, 2010).

Mosquitoes were knocked down with CO_2 and transferred to each bottle (5 mosquitoes/bottle). We recorded the living mosquitoes every 5 minutes until all were dead. The sign of death is that mosquito cannot stand or fly. (Control and Prevention, 2010).

Eight insecticides (malathion, permethrin, resmethrin, β-cyfluthrin, chlorpyrifos, deltamethrin, etofenprox, and fenitrothion) were used in the adult bioassay to test the actual time to 100% mortality of the *Aedes albopictus* from Birmingham, Dothan, Mobile, Montgomery, Tuscaloosa, and Tuskegee. All adult bioassays were repeated at least four times with five mosquitos each time.

4.3.4 Larval bioassay

Acetone was used as the solvent when preparing the stock and serial dilutions of all insecticides. Ten fourth instar larvae of similar size were placed in a 20 ml bottle (20 ml disposable scintillation vials VWR®) with normal chlorinated water and 1% insecticide solution in acetone at the required concentration. Three to four concentrations were prepared to make sure the mortality of the mosquitoes ranged from 0-100%. The control group received only 1% acetone. All the tests were incubated at 25°C, recorded after 24 h and repeated at least three times on different days. Polo-PC software was used to assess the bioassay data and construct the probit analysis; mortality was controlled according to Abbott's correction (Liu et al., 2004)

4.3.5 Analysis

Averages and differences were calculated with Excel. The result of the larval bioassay was analyzed by POLO through probit analysis.

4.4 Result

4.4.1 Adult bioassay of *Aedes albopictus* in Alabama from 2017 to 2018

According to the results, *Aedes albopictus* can survive longer than the diagnostic time in fenitrothion, permethrin, and resmethrin. Resmethrin is the weakest insecticide, and the tested mosquitos were able to survive eight more minutes than the diagnostic time. Permethrin, was the next weakest, with the mosquitoes surviving for seven minutes longer than the diagnostic time, and fenitrothion, was next, surviving for six more minutes than the diagnostic time. By contrast, the remaining insecticides, chlorpyrifos, deltamethrin, etofenprox, malathion, and β-cyfluthrin can kill all tested mosquitos in less than the diagnostic time. Chlorpyrifos and β-cyfluthrin are the strongest insecticides, and these were able to kill all tested mosquitos four minutes before the diagnostic time. However, *Aedes albopictus* from Mobile can struggle almost one minute longer than the diagnostic time (**Figure 19 and 21**). Among the six cities, the *Aedes albopictus* forms in Mobile and Tuscaloosa are the most tolerance strains, showing the highest survival times compared to this species from other locations. The *Aedes albopictus* from Birmingham strain is the most sensitive strain that shows the lowest survival times compared to the others (**Figure 20**). Similarly, *Aedes albopictus* from Mobile shows the most powerful ability to endure resmethrin, with mosquitoes struggling for 12 more minutes than the diagnostic time; *Aedes albopictus* from Birmingham is the most susceptible strain, and these mosquitoes died 19 minutes before the diagnostic time (**Figure 19**).

4.4.2 Fenitrothion and permethrin larval bioassay of the *Aedes albopictus* collected in Tuscaloosa, Tuskegee, Dothan, and Montgomery.

To investigate the resistance status further, fenitrothion and permethrin were chosen for use in the larval bioassay because these two insecticides can enable tested mosquitoes to live longer than the diagnostic time. Fenitrothion and permethrin belong to different classes of insecticide—organophosphate, and pyrethroids, respectively. According to the result, fenitrothion is highly toxic to *Aedes albopictus* with an LC50 value of approximately 0.02 and LC90 of approximately 0.045 (**Figure 23, 24**). Permethrin is less toxic to the *Aedes albopictus,* showing an LC50 value of approximately 0.1 and an LC90 value of approximately 0.25 (**Figure 23, 24**). Organophosphate is still more toxic than pyrethroids. Additionally, the Montgomery strain shows the highest slope with fenitrothion (7.825) and the lowest slope with (2.837) in permethrin. By contrast, the Tuskegee strain shows the highest (6.132) slope in permethrin. The remaining strains have a similar slope with the two insecticides (**Table 7**).

4.5 Discussion

Scourge (active ingredients: Resmethrin), anvil (active ingredients: Sumithrin), permethrin and malathion are the insecticides most widely used to control the mosquitoes that carry disease. However, according to our results, we recommend using β-cyfluthrin and deltamethrin instead of permethrin, resmethrin, and malathion.

Permethrin and resmethrin are two insecticides in pyrethroids that focus on the central nervous system, can block the sodium channel, prolong its opening time, and cause hyperexcitation until death (Gilbert and Gill, 2010; Sparks and Nauen, 2015). However, according to our result, permethrin and resmethrin are two insecticides that can allow tested mosquitoes survive for almost 10 minutes longer than the diagnostic time. Permethrin has been used widely in Alabama for 15 years (Liu et al., 2004). The long-term and inappropriate using of the same insecticide can increase the risk that insecticide resistance will develop (Feng et al., 2018; Liu, 2015).

Scientists have proposed several mechanisms for insecticide resistance (Moyes et al., 2017; Naqqash et al., 2016). The first mechanism is an insensitive target, such as those resulting from mutations in the sodium channel (L-F, H-S), which can change the property of the sodium channel, making it less sensitive to pyrethroids or DDT (Dong, 2007; Liu, 2015). The second mechanism is the overexpression of detoxifying enzymes, which can increase the volume of P450, GST, carboxylesterases or other detoxifying enzymes to hydrolyze or conjugate the insecticide compound, with the insecticide being excreted or allowed to bind to a fat body, such as those overexpressed in the CYP4, CYP6, CYP9 and CYP12 family (Ishak et al., 2016; Liu et al., 2011; Reid et al., 2014). Other mechanisms include behavior avoidance (Chareonviriyaphap et al., 2013) and penetration reduction (Bass and Jones, 2016; Plapp, 1976).

Malathion and chlorpyrifos also have the high toxicity to mosquitoes according to our results. However, we do not suggest the use of these two insecticides. These insecticides are organophosphates that inhibit AchE so that Ach cannot be hydrolyzed but remains bound to AchR, causing hyperexcitation to death (Sparks and Nauen, 2015). However, this powerful insecticide can also be poisonous to humans—3 million poisonings, with two hundred thousand deaths, have been attributed to organophosphates (Eddleston et al., 2008)—and can be detected worldwide in various substrates, including air, water, food and tissue of humans or animals, among others. (Bhushan et al., 2003). Repeated and prolonged exposure to organophosphates may result in impaired memory, severe depression, confusion, headache, speech difficulties, sleepwalking and so on. Even the low-level exposure can also do damage to the brain development (Eskenazi et al., 1999)

The two insecticides we suggest—β-cyfluthrin and deltamethrin—belong to the pyrethroids, which are safe to humans (Wolansky and Harrill, 2008) but still highly toxic to mosquitoes compared to others insecticides, such as permethrin and deltamethrin.

Mobile not only is the most suitable city for mosquitos to live, but it also has mosquitoes with a high tolerance for insecticide. Perhaps, because Mobile is a seaport and highly urbanized city, it provides a good source of blood for mosquitoes (Carbajo et al., 2006; Ruiz et al., 2004); furthermore, this city has warmer temperatures compared to the other places in Alabama, thereby providing a longer time for mosquito to survive and breed to generate offspring (Delatte et al., 2009)

The *Aedes albopictus* collected from Montgomery shows a more heterozygous response to permethrin and has the highest LC50 and LC90 values. Perhaps, the wide overuse of permethrin in Montgomery killed most of the susceptible mosquitoes and let the resistant mosquitoes breed the next generation, which may then exhibit a greater ability to withstand insecticide. Compared to its response to the permethrin, the *Aedes albopictus* collected from Montgomery showed a steep slope with the fenitrothion, which indicates that a much larger portion of the field population of this strain was homozygous to the fenitrothion. By contrast, the *Aedes albopictus* collected from Tuskegee strain shows a more homozygous response to permethrin than this species at other locations. Tuskegee is a small city near the forest, so the residents do not need to worry as much about mosquito-borne disease as those living in large cities (Obenauer et al., 2017; Russell et al., 2002); furthermore, the forest can help colonies of other mosquitoes, such as *Aedes bahamensis*, which do not need blood to breed the next generation (O'Meara et al., 1993). Therefore, because the residents of Tuskegee need insecticide less frequently, the mosquitoes do not develop resistance to insecticide as quickly.

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Table 3: Diagnostic time and diagnostic concentration based on CDC protocol.

Insecticide	**Diagnostic dose (µg/µl)[a]**	**Diagnostic time (mins)[b]**
Chlorpyrifos	0.1	45
Deltamethrin	0.00375	30
Etofenprox	0.0625	30
Malathion	0.25	30
Permethrin	0.075	15
Resmethrin	0.15	10
Fenitrothion	0.25	30
β-cyfluthrin	0.05	30

[a] Diagnostic dose is a dose of insecticide that kills 100% of susceptible mosquitoes within a given time.

[b] Diagnostic time is accepted time for the insecticide to kill 100% of susceptible mosquitoes.

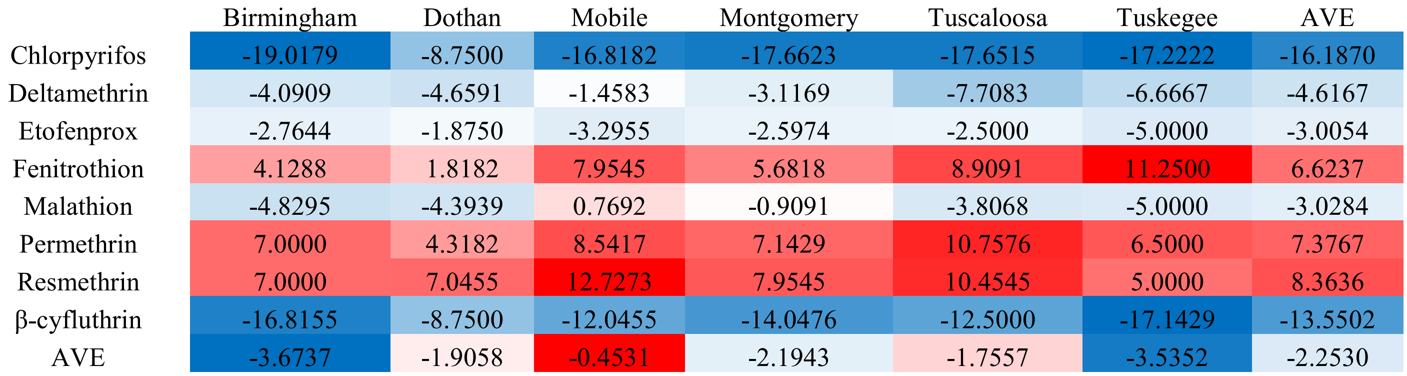
Table 4: Fenitrothion and Permethrin larval bioassay of the *Aedes albopictus* collected in Tuscaloosa, Tuskegee, Dothan, and Montgomery

Insecticide	**Location**	**df**	**χ^2 [a]**	**LC50[b](CI)[c]**	**LC90[b](CI)[c]**	**n**	**slope**
Fenthion	Tuscaloosa	5	19.161	0.01230 (0.00642-0.01954)	0.02998 (0.01901-0.12935)	211	3.312+-.417
	Dothan	3	3.3561	0.01925 (0.01492-0.02507)	0.03771 (0.02801-0.08113)	184	4.389+-.658
	Montgomery	3	4.7156	0.01779 (0.01298-0.02285)	0.02594 (0.02058-0.04429)	168	7.825+-1.124
	Tuskegee	3	2.9701	0.03029 (0.02487-0.03797)	0.05989 (0.04590-0.09715)	106	4.328+-.770
Permethrin	Tuscaloosa	5	14.743	0.11892 (0.08948-0.15783)	0.26683 (0.19284-0.50934)	329	3.651+-.338
	Dothan	5	2.0761	0.08013 (0.06730-0.09682)	0.14797 (0.11728-0.23288)	144	4.811+-.932
	Montgomery	4	1.0198	0.16487 (0.13054-0.20699)	0.46650 (0.34749-0.73705)	178	2.837+-.397
	Tuskegee	3	3.4203	0.05777 (0.04203-0.07864)	0.09347 (0.07078-0.20214)	127	6.132+-.1.057

[a] Person $\chi^{2,}$ goodness-of-fit test.

[b] LC_{50} and LC_{90} values in ppm

[c] 95% confidence interval, toxicity of insecticide is considered significantly different when the 95% CI fail or overlap.



	Birmingham	Dothan	Mobile	Montgomery	Tuscaloosa	Tuskegee	AVE
Chlorpyrifos	-19.0179	-8.7500	-16.8182	-17.6623	-17.6515	-17.2222	-16.1870
Deltamethrin	-4.0909	-4.6591	-1.4583	-3.1169	-7.7083	-6.6667	-4.6167
Etofenprox	-2.7644	-1.8750	-3.2955	-2.5974	-2.5000	-5.0000	-3.0054
Fenitrothion	4.1288	1.8182	7.9545	5.6818	8.9091	11.2500	6.6237
Malathion	-4.8295	-4.3939	0.7692	-0.9091	-3.8068	-5.0000	-3.0284
Permethrin	7.0000	4.3182	8.5417	7.1429	10.7576	6.5000	7.3767
Resmethrin	7.0000	7.0455	12.7273	7.9545	10.4545	5.0000	8.3636
β-cyfluthrin	-16.8155	-8.7500	-12.0455	-14.0476	-12.5000	-17.1429	-13.5502
AVE	-3.6737	-1.9058	-0.4531	-2.1943	-1.7557	-3.5352	-2.2530

[a] number was calculated by actual 100% mortality time minus diagnostic time.

Figure 13: Difference between actual time for 100% mortality and the diagnostic time

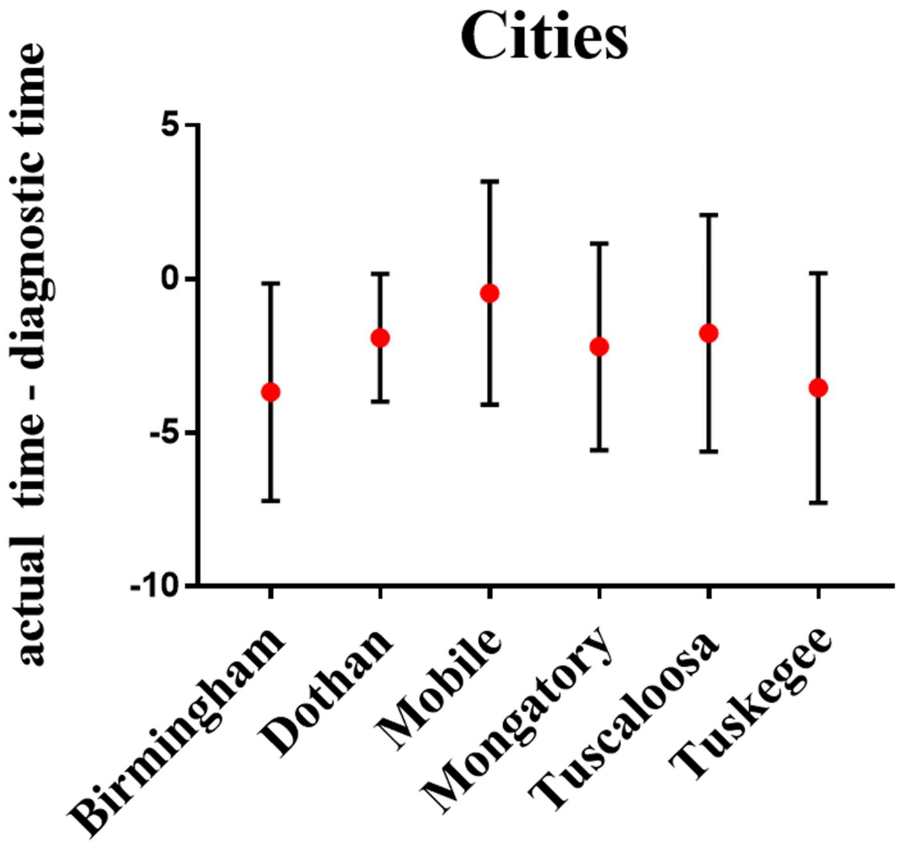


Figure 14: Difference in the actual mortality time and diagnostic time in different cities

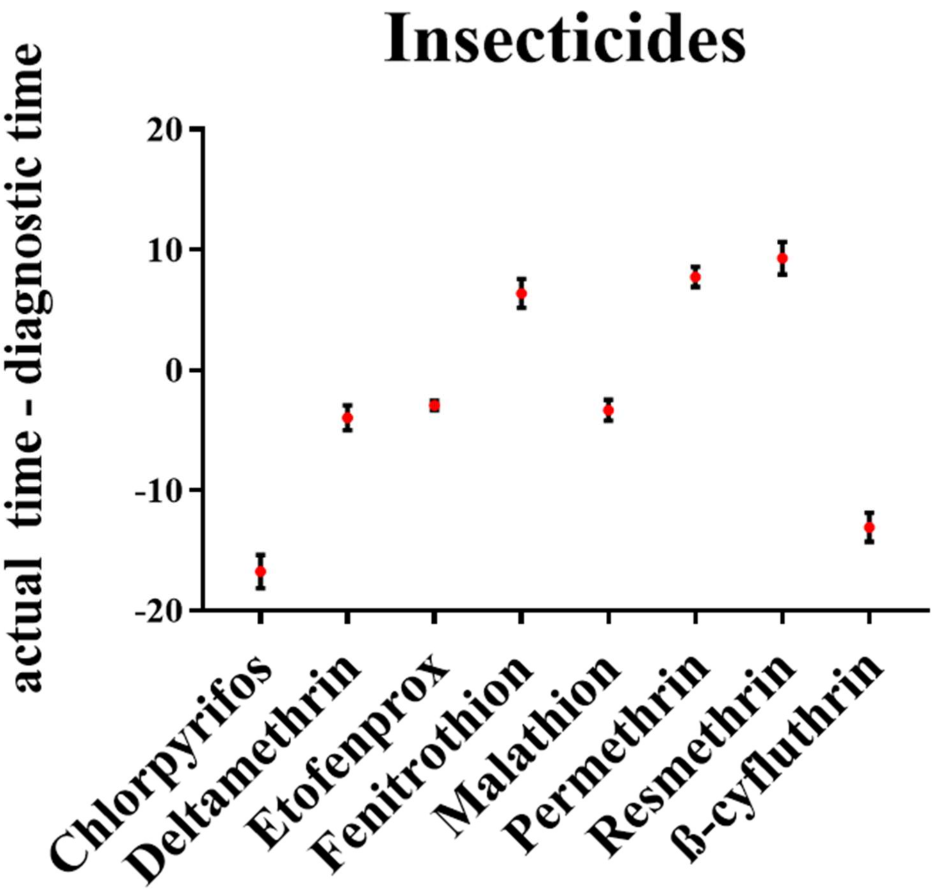


Figure 15: Difference of actual mortality time and diagnostic time in different insecticides

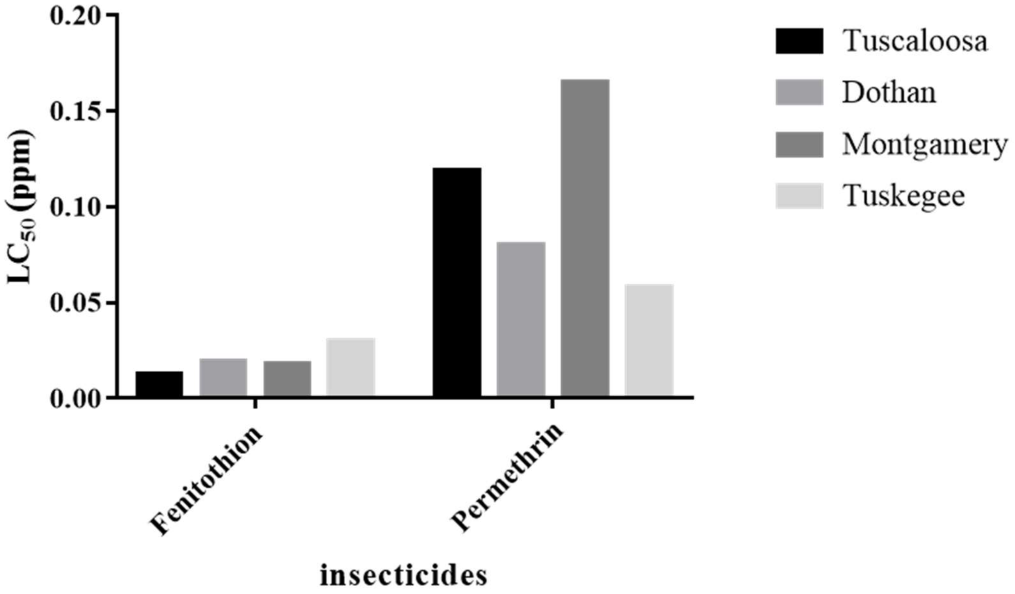


Figure 16: LC50 of Fenthion and Permethrin in Tuscaloosa, Dothan, Montgomery and Tuskegee

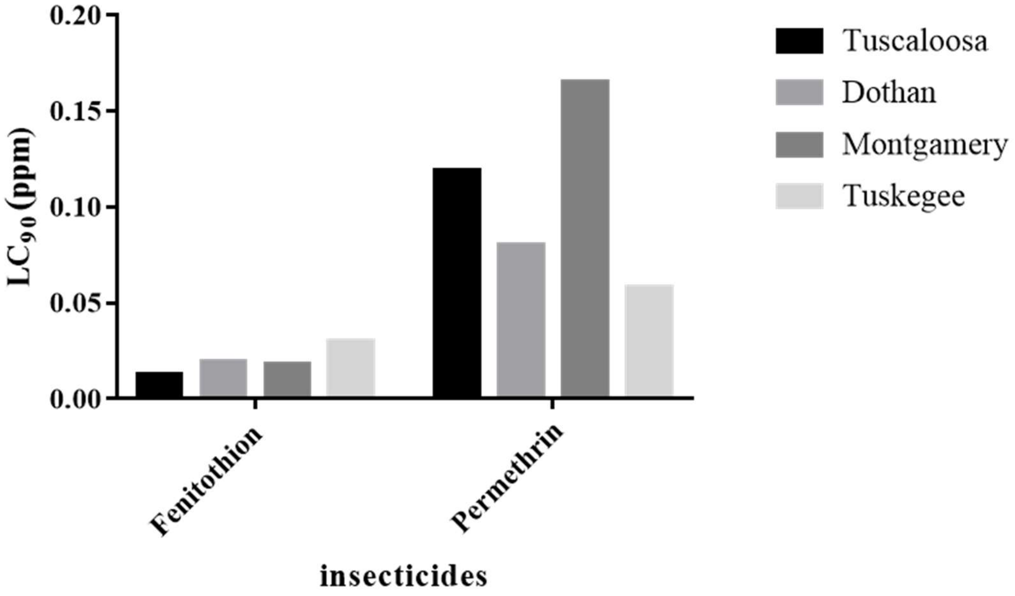


Figure 17: LC90 of Fenthion and Permethrin in Tuscaloosa, Dothan, Montgomery, and Tuskegee.

Chapter 5: Future Study

5.1 Investigating the dynamic population of *Culex quinquefasciatus* and the difference in species diversity between forest and city

5.1.1 Introduction

Aedes transmits serious viruses, such as Zika virus, dengue virus, chikungunya virus and other serious viruses, resulting in several outbreaks in the past few years (Paupy et al., 2009): chikungunya in August 2007 in Italy (Angelini et al., 2008) and Zika in February 2016 in Brazil (Organization, 2016). *Culex* can also carry diseases and spreads them all over the world; these include the West Nile virus, St. Louis encephalitis, periodic lymphatic filariasis, Japanese encephalitis and other diseases (Farid et al., 2001). West Nile fever, a disease transmitted between birds and mosquitoes caused 16,169 cases and 1,549 deaths during the years 1999-2012 in the United States (Petersen et al., 2013) and has spread throughout Asia, the Middle East, Southern Europe, Western Russia, Southeastern Asia and Australia (Petersen et al., 2013). Japanese encephalitis occurs in both Asia and the Pacific, and results in 30,000-50,000 cases every year (Erlanger et al., 2009). Unlike *Aedes*, *Culex* will lay their eggs directly on the surface of the water.

According to our previous observation, three mosquito species are found in Tuskegee, which is near the forest; and only 1 species was found in Mobile, which is a highly urbanized seaport city; therefore, we hypothesize that more mosquito species will occur in the forest than in the city.

5.1.2 Method and material

5.1.2.1 Site collection:

Tuskegee, Mobile, Tuscaloosa, Montgomery, Birmingham, and Dothan will be used for trapping, as in the previous year. Two traps will be placed in each city: one will be away from active residential areas and placed in forests, gardens or mountains; the other trap will be placed near the residential area in parks, drains or at tire stores.

5.1.2.2 *Culex* identification:

In *Culex spp.*: The proboscis is longer than the maxillary palpus. The scales of wings are long and narrow. The proboscis is longer than the antenna, flagellomere1 about as long as flagellomere2.

In *Culex quinquefasciatus*: bands or lateral patch of pale scales along the basal border on abdominal terga; Scutum have mid-dorsal setae.

5.2 Functional study of the overexpressed gene involved in insecticide resistance

5.2.1 Introduction

The inappropriate overuse of insecticides stimulates the mosquito to develop insecticide resistance, which has become one of the major barriers to mosquito-borne disease control. *Aedes aegypti* has been confirmed to have resistance to varieties of insecticides, including temephos, malathion, deltamethrin, permethrin, DDT, chlorpyrifos and propoxur, worldwide (Chen et al., 2016; Kamgang et al., 2017; Li et al., 2018; Marcombe et al., 2014; Ponlawat et al., 2005); Compared to *Aedes aegypti*, *Aedes albopictus* is less resistant but has still been reported to resist three major classes of insecticides: chlorpyrifos, pyrethroids, and carbamate, worldwide (Goindin et al., 2017; Ishak et al., 2015; Rodríguez et al., 2007; Seixas et al., 2017). The insensitivity of targets that show the overexpression of detoxifying enzymes, behavioral avoidance and penetration reduction have been confirmed to be involved in the insecticide resistance. P450 is the major detoxifying enzyme that degrades the toxicity of insecticides and has been proven to be involved in insecticides through overexpression (Liu et al., 2011; Reid et al., 2014). Previous studies have demonstrated some overexpressed genes in *Aedes albopictus*, but their function is still unclear (Ishak et al., 2016).

The high protein production efficiency and the eukaryotic-mediated foreign gene of the baculoviral-mediated foreign gene expression system cause more scientists to apply this system to investigations of the function of this specific gene. The constructed recombinant baculovirus sends foreign genes into the host cells, and the large-scale expression of interested proteins by cells infected by recombinant baculovirus are two key elements in the baculoviral system (Feng and Liu, 2018). As a high-throughput colorimetric method, the tetrazolium salt-based assay (MMT) will be used to measure cell viability. Only living cells can metabolize the yellow MTT reagent to a dark purple formazan precipitate, which can be colorimetrically analyzed after being dissolved in organic solvents (Stockert et al., 2012).

5.2.2 Method

5.2.2.1 RNA extraction, cDNA preparation, and Quantitative Real-Time Polymerase Chain Reaction

Total RNA will be extracted from susceptible and resistant strains. Genomic DNA will be degraded before cDNA is synthesized with a TURBO DNA-free kit (Ambion) according to the manufacturer's instructions. cDNA will be synthesized by 5x iScript™ Universal SYBR and a CFX 96 Real Time system.

5.2.2.2 Recombinant baculoviral expression of the specific gene

The specific gene can be directly cloned into pENTR™ TOPO vector with CACC added at the 5' end forward primer and overhang sequence GTGG in the vector. The recombinant vector will be transformed into One Shot® competent E. coli and purified using the E.Z.N.A plasmid DNA Mini Kit. *Spodoptera frugiperda* (sf9) cell was incubated in the solutions of SF900-III medium containing 10% FBS and Grace's Insect medium. The recombinant baculovirus, which was transferred into sf9, will be used to generate living cells with recombinant baculoviral and interested protein.

5.2.2.3 MTT assay

Control cells and infected cells will be treated with permethrin in concentrations ranging from 12.5 to 400 μm at 48 h postinfection. After 48 h incubation in 37˚C, the medium will be replaced by PBS buffer and 200 μl MTT for 4 h °incubation in 37°C. OD (optical density) number at 540 nm will be read by Cytation 3 Cell Imaging Reader to calculate the cell viability.

5.3 Using CRISPR-cas9 to investigate the GPCR-regulatory pathway.

5.3.1 Introduction

Clustered Regularly Interspaced Short Palindromic Repeats – CRISPR is the most rapidly developing technology in the current generation of genome editing technology, next to meganucleases, ZFN (zinc finger nuclease) and TALEs (transcription activator-like effectors). CRISPR-cas9 will be used widely in the future because it can target any site on the genome with a short RNA guide (Hsu et al., 2014). CRISPR was found in the immune system in bacteria first and has been investigated for more than a decade. Early in 1987, a strange set of 29 nt repeats downstream of the iap gene has been reported by Nakata and their colleagues (Ishino et al., 1987). In the subsequent 10 years, Mojica and colleagues eventually found that these interspaced repeat sequences exist in a variety of organisms and named it

CRISPR (Mojica et al., 2000). Cas9 protein, as the most broadly used protein in CRISPR genome editing has been found at the type II CRISPR immune system at the beginning; it can bind to the repeat unique gene sequence on the genome and help to locate the target sequence (Platt et al., 2014; Ran et al., 2013).

To initiate this genome editing process, transactivating crRNA (tracrRNA) will bind with crRNA to form guide RNA. After that, the RNA complex has been established, the cas9 protein will be established as the target sequence. Eventually, the target sequence will be cut with the assistance of RNase III (Doudna and Charpentier, 2014). Until now, CRISPR-Cas9 has been successfully adapted in a variety of organisms, be they human cells, mice, monkeys, mosquitoes, houseflies, and different plants and bacteria (Li et al., 2017; Niu et al., 2014; Platt et al., 2014; Shalem et al., 2014; Sharma et al., 2017).

G-protein-coupled receptors (GPCRs) are famous for extracellular signal transduction and intracellular second messenger regulation. Interestingly, GPCR regulation pathways can regulate the expression of P450, which is related to insecticide resistance (Li et al., 2015; Li and Liu, 2017). Knocking out the gene of G-protein, cAMP, protein kinase A or other effectors that play a role in GPCR regulation pathways can offer a more convenient way to observe the following changes in physiology, insecticide resistance or other systems.

5.3.2 Method

CRISPR-cas9 primer will be designed by CRISPR RGEN Tools, synthesized and purified by Ambion MegaScript T7 kit and MEGAclear™ Kit Purification for Large-Scale Transcription Reactions, respectively.

5.4 Reference

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Chapter 6 Conclusion and Summary

During this research, three mosquito species were identified: *Aedes albopictus, Aedes triseriatus,* and *Aedes japonicus*. *Aedes albopictus* was recognized as the primary species, whereas *Aedes japonicus* was recognized as the pioneer species. According to our research, Mobile attracts mosquitoes easily and has a large proportion of resistant strains, which need to be treated quickly. β-cyfluthrin and deltamethrin are the two insecticides we suggest because they are less toxic to humans than malathion and fenitrothion and more toxic to mosquitoes than permethrin and resmethrin, which can be tolerated by mosquitoes. Additionally, we need to be aware in April and May, when the temperature reaches up to 57°F, that mosquitoes will emerge and breed. August is a good month to treat mosquitoes because the populations are large during this month. Biocontrol and gene-driven alternatives to insecticide use can also be used to control the population and prevent mosquito-borne diseases. For example, *Wolbachia* has been used to control mosquito populations, CRISPR-cas9 has been used to reduce the risk of mosquitoes carrying a virus, and essential oil has been used to treat mosquitoes that have found to be resistance to pyrethroids.