THESIS

# PERMETHRIN RESISTANCE STATUS AND ASSOCIATED MECHANISMS IN *AEDES ALBOPICTUS* MOSQUITOES: IMPLICATIONS FOR VECTOR CONTROL

Submitted by

Ashley J. Janich

Department of Microbiology, Immunology and Pathology

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Spring 2020

Master's Committee:

Advisor: Rebekah C. Kading

Brian D. Foy
Franck E. Dayan

Copyright by Ashley J. Janich 2020

All Rights Reserved

ABSTRACT

PERMETHRIN RESISTANCE STATUS AND ASSOCIATED MECHANISMS IN *AEDES ALBOPICTUS* MOSQUITOES: IMPLICATIONS FOR VECTOR CONTROL

There are major public health concerns regarding the spread of mosquito-borne diseases such as dengue, Zika, and chikungunya. The primary method to reduce the spread of these diseases is by killing the major mosquito vectors, *Aedes aegypti* and *Aedes albopictus*, with insecticides. However, with continued use of insecticides, these species have started developing resistance to the toxic effects of insecticides and higher concentrations and doses are needed to continue to effectively control these vectors. One of the insecticides that is frequently used to control these mosquitoes is permethrin, a type I pyrethroid, due to its rapid knock down effect and its low toxicity to mammals and other vertebrates. Resistance has been extensively studied in *Ae. aegypti* from various countries around the world. However, there are comparatively fewer studies that have examined resistance in *Ae. albopictus* mosquitoes, especially in the Americas. In this study, we wanted to investigate the permethrin resistance status of *Ae. albopictus* mosquitoes collected from Southern Mexico and Southern Texas. Additionally, we also wanted to test if permethrin resistance could be artificially selected in the laboratory over several generations. Lastly, we wanted to investigate the potential mechanisms that have been found to previously confer resistance in other mosquitoes. The two mechanisms that were investigated were knock down resistant (*kdr*) mutations, specifically the F1534C mutation of the voltage gated sodium channel (VGSC) gene, and the activity of metabolic enzymes that break down and detoxify insecticides.

We found that there were low levels of resistance in our populations of *Ae. albopictus* from Mexico and Texas, with resistance ratios (RRs) ranging from 1.18 to 2.40 relative to our control strain, ATMNJ95. The results of our selected strain were unexpected. There was a gradual increase in the RR over several generations of selection, but our last selected generation had a lower RR compared to the previously selected generations. We did not find the F1534C mutation in any of our *Ae. albopictus* strains from Mexico. There was significantly higher cytochrome P450 activity in our permethrin-selected and non-selected strains of *Ae. albopictus* from Mexico compared to our control strain. The same pattern was also observed in the counterpart *Ae. aegypti* strains that were analyzed for comparison. Our results suggest that *Ae. albopictus* from the regions we sampled are still mostly susceptible to pyrethroids. However, it is possible we are observing beginning stages of resistance in this species. Alternatively, we also acknowledge the possibility that there might be ecological or historical differences between *Ae. albopictus* and *Ae. aegypti* that have resulted in different levels of resistance between the two species. It is important to continue monitoring populations of *Ae. albopictus* in order to prevent the development of resistance in the future.

## ACKNOWLEDGEMENTS

There are many people I would like to thank who have helped make this work possible. First and foremost, I would like to thank my original advisor, Dr. William Black IV, for giving me the opportunity to be a student in his lab, first as an undergraduate, and then again as an M.S. student. Dr. Black's mentorship and guidance have greatly influenced my education here at Colorado State University. I am very fortunate and grateful to be able to learn from him, not only as a professor during my undergraduate and graduate years, but also by having him as my advisor. I have always been amazed by his depth of knowledge, and I am lucky that I was able to learn everything from him that I could while working alongside him. Secondly, I would like to thank Dr. Rebekah Kading, for taking over as my advisor and helping me finish my thesis when unexpected circumstances arose. I cannot thank her enough for all of the mentorship, guidance, and feedback that she gave me throughout this process. Additionally, I would also like to thank the other members of my committee, Dr. Franck Dayan and Dr. Brian Foy for all their valuable help and feedback on my thesis writing and for their mentorship throughout this process as well. I have been so fortunate to be able to work with all of these amazing professors/scientists and to have them as my committee members.

I would also like to thank other members of the Black lab, especially Dr. Karla Saavedra-Rodriguez and Dr. Farah Vera-Maloof, not only for the work they contributed to helping me with this project, but also for their support, guidance and friendship throughout my time spent working in the lab with them. On that same note, I would also like to thank Dr. Selene Garcia-Luna and Dr. Corey Campbell for their support and help throughout this project. I consider these women, along with Dr. Kading, to be role models for myself and other women in science. I hope

that, like these women, I will be able to contribute to the scientific community and aspire other young women to do the same.

I would also like to thank all of the undergraduate students of the Black lab that assisted with this project by helping with mosquito rearing and DNA extractions over the years: Amaury Rodriguez, Kyra Headrick, Lizzy Havlik, Alana Kim, Maeve Kelly, and Abby Jackson, thank you so much for all your help!

I also would like to thank our collaborators at Centro Regional de Investigación en Salud Pública (CRISP) in Tapachula, Chiapas, Mexico. I especially would like to thank Dr. Americo Rodriguez, Dr. Paty Penilla-Navarro, Alma Delia Lopez-Solis, and Francisco Solis-Santoyo for their involvement and support with this project.

On a more personal note, I of course owe a lot of thanks to my loving parents, Aimee and Tom Janich, as well as my little brother, James Janich, for their constant love and support, not only throughout my graduate school education, but throughout my life. I cannot thank you three enough for always being there for me and for helping me achieve this milestone. I love you all so much!

I would also like to thank my friends who have been here for me throughout this process and have helped me through my struggles simply by being there for me. I have a huge amount of gratitude for my generous friend, Ryan Evans, who let me borrow his laptop during my last year of graduate school so that I could use it to write this thesis (since my laptop decided to break at the worst time!). I also want to thank my other wonderful friends who provided emotional support and fun times to keep me going when the going got tough, especially Stephanie Bartmann, Patrick Dorsey, and Enrique Diaz. I would also like to thank my other friends that

were there for me and supported me through this process, including Dr. Anna Fagre, Matt Robinson and Eric Kelly. Thank you all so much!

I also owe special thanks to Dr. Kristina Quynn, founder of CSU Writes, for all of the amazing and extremely useful, resources that the writing program offered. I especially want to thank her for designing and coordinating the writing retreat sessions for graduate students. I lost track of how many I attended, but it is fair to say that a huge chunk of this thesis was written at those retreats! Not only did the retreats help me set aside time to focus solely on my thesis, but Kristina always provided valuable information for how to write more efficiently and how to conquer the challenges we face while writing long pieces of work, like this thesis. I owe her a huge thanks, as well as all of the volunteers and interns who helped with the CSU writing events. Thank you so much!

And lastly, I owe a huge amount of gratitude to Heidi Runge, the Academic Support Coordinator for Graduate Studies in the MIP department. Heidi is phenomenal in her ability to support graduate students by guiding us through all the frustrating processes and paperwork that we must complete in order to graduate, and for always being available to answer any questions/concerns we have. We all would be truly lost without you, Heidi!

Of course, there are so many other individuals who deserve thanks and praise for helping me achieve this milestone: past professors, teachers, educators and mentors throughout my entire education (from grade school to graduate school), all the people working behind the scenes that make our work and research at CSU possible, and so many other friends and family members who have been part of my life. Thank you all so much!

All my love and appreciation,

Ashley (a.k.a. A.J.)

## DEDICATION

This thesis is dedicated to my grandparents, “Grammy” Myrna Donaho, “Papa” James Donaho, “Grammy” Dorothy Janich and “Papa” George Janich.

I am here today because of all of you, and the loving families you created.

I love you all so much.

TABLE OF CONTENTS

ABSTRACT ... ii
ACKNOWLEDGMENTS ... iv
DEDICATION ... vii
CHAPTER 1 – LITERATURE REVIEW ... 1
Geographical Distribution and Dispersal of *Aedes aegypti* and *Aedes albopictus* ... 1
History of Vector Control in the Americas Prior to Arrival of *Aedes albopictus* ... 4
Biology, Ecology and Behavior of *Aedes albopictus* ... 7
Vector Competence and Capacity of *Aedes albopictus* ... 11
Impacts of Disease Caused by *Aedes* Mosquitoes ... 14
Arbovirus Disease Prevention Strategies and the Rising Problems with Insecticide Resistance ... 15
Pyrethroid Insecticides: What, Why and How? ... 21
Insecticide Resistance Mechanisms ... 25
Aims and Hypotheses of this Study ... 26
CHAPTER 2 – INVESTIGATION OF PERMETHRIN RESISTANCE IN *AEDES ALBOPICTUS* MOSQUITOES ... 28
Introduction ... 28
Materials and Methods ... 29
Mosquito Collections ... 29
Bottle Bioassays ... 34
Statistical Analysis of Bottle Bioassays ... 37
Artificial Permethrin Selection ... 37
Results ... 38
Baseline Permethrin Resistance of *Aedes albopictus* from Mexico and Texas ... 38
Artificial Permethrin Selection ... 39
Discussion ... 42
Low Levels of Resistance in Field *Aedes albopictus* Mosquitoes ... 42
Permethrin Selection ... 47
CHAPTER 3 – INVESTIGATION OF POSSIBLE MECHANISMS CONFERRING RESISTANCE IN LESS SUSCEPTIBLE *AEDES ALBOPICTUS* POPULATIONS ... 53
Introduction ... 53
Materials and Methods ... 58
Mosquitoes ... 58
Sequencing and Genotyping *kdr* Mutations ... 58
Biochemical Assays ... 65
Statistical Analysis of Biochemical Assays ... 69
Results ... 70
Sequencing and Genotyping *kdr* Mutations ... 70
Biochemical Assays ... 70
Discussion ... 78
Sequencing and Genotyping *kdr* Mutations ... 78
Biochemical Assays ... 80

CHAPTER 4 – SUMMARY AND FUTURE DIRECTIONS....................................................84
Summary.......................................................................................................................84
Resistance Monitoring and Management: Limitations and Areas Needing Improvement 87
Future Directions .........................................................................................................89
REFERENCES ..................................................................................................................93
APPENDIX: SUPPLEMENTAL INFORMATION .................................................................116
Additional Information on Mosquitoes Collected from Chiapas, Mexico .....................116
Additional Information on Mosquitoes Collected from Weslaco, Texas .......................116
Supplemental Tables.....................................................................................................117

# CHAPTER 1 – LITERATURE REVIEW

## Geographical Distribution and Dispersal of *Aedes aegypti* and *Aedes albopictus*

*Aedes* (*Stegomyia*) *aegypti* (Linnaeus 1762) and *Aedes albopictus* (Skuse 1895) mosquitoes both live in tropical and subtropical regions around the world. However, *Ae. albopictus* have been found in areas further north than *Ae. aegypti* due to their ability to undergo diapause during colder temperatures (Paupy et al. 2009). For example, in Japan *Ae. albopictus* have been found just north of 40° in latitude (Mogi and Tuno 2014) and even as far north as New Hampshire in the United States (U.S.) (Hahn et al. 2017). Generally speaking, *Ae. aegypti* is more closely associated with urban areas where human populations are condensed and artificial containers from humans are abundant. While *Ae. albopictus* have been more commonly thought to prefer rural or suburban habitats where vegetation and natural breeding containers are more prevalent. However, there are instances when distributions of these species overlap and they can be found in the same habitats and geographical regions, and can even share aquatic breeding containers, as observed with the mosquitoes collected for the present study.

But despite having similar geographical distributions, the two species differ in their historical background as to how and when they came to North and South America. The *Ae. aegypti* commonly known today is the domesticated species, *Aedes aegypti aegypti*, which originated in Africa from a more ancestral species, *Aedes aegypti formosus* (Tabachnick 1991, Brown et al. 2014). *Aedes aegypti* is believed to have been first transported to the Northern/Southern Americas by means of slave trade ships between the 1400s and 1700s (Tabachnick 1991, Brown et al. 2014).

On the other hand, *Ae. albopictus* is relatively new to the Americas. Originally *Ae. albopictus* was initially only native to Asia (Benedict et al. 2007) but eventually dispersed to other countries around the world through the international trade of used tires (Sprenger and Wuithiranyagool 1986, Reiter 1998, Benedict et al. 2007) and lucky bamboo (Hofhuis et al. 2009). By the mid-1980s, *Ae. albopictus* was established in both Brazil and the U.S. (Reiter 1998, Benedict et al. 2007) and was later established in parts of Mexico (M.X.) in the late 1980s (Benedict et al. 2007). By comparison, *Ae. aegypti* has been present in the New World for a much longer period, while *Ae. albopictus* has been here for much less time and is considered an invasive species here and in many other regions of the world.

When considering the mosquitoes we tested for this study, which were collected from Southern M.X. and Southern Texas, it is important to review when and how *Ae. albopictus* became established in these regions. Technically speaking, the U.S. acquired its first established breeding populations of *Ae. albopictus* when Hawaii became an official state in 1959, although the species had been established on the islands since the end of the 19$^{th}$ century (Joyce 1961). The first established breeding population of *Ae. albopictus* in the continental U.S. was reported by Sprenger and Wuithiranyagool (1986) after finding the species in numerous collections in and around Houston, Texas in 1985. From this study they speculated that *Ae. albopictus* had been in the region for possibly months or years due to the widespread distribution and high abundance relative to other mosquito species that were collected. However, since there were no *Ae. albopictus* detected in a previous survey of the same county in 1980, the authors surmised that the species likely became established in the region sometime after that (Sprenger and Wuithiranyagool 1986). *Aedes albopictus* had been collected in the U.S. three times prior to this in 1946, 1971 and 1983 (Pratt et al. 1946, Eads 1972, Reiter and Darsie 1984), but with no

evidence of breeding populations being established (Sprenger and Wuithiranyagool 1986), which means it could be possible that *Ae. albopictus* became established earlier in certain parts of the U.S. prior to actual sampling data. The discovery of breeding populations of *Ae. albopictus* in Texas prompted subsequent surveillance in the surrounding areas, and by 1986 the species was detected in Louisiana, Tennessee, Mississippi, Florida, Alabama, Missouri and other counties near Houston, Texas (Moore et al. 1988, Reiter 1998). That same year, *Ae. albopictus* was detected in Brazil for the first time (Forattini 1986). Additional surveillance in 1987 showed that *Ae. albopictus* was less abundant (relative to other container-breeding *Aedes* spp.) as distance from Houston increased (Moore et al. 1988), which further supports the hypothesis that this was one of the first regions in the U.S. to be infested with *Ae. albopictus*. Genetic analysis of *Ae. albopictus* that were sampled shortly after their introduction into the U.S. and Brazil strongly suggested that the populations of *Ae. albopictus* in both countries originated from Japan (Kambhampati et al. 1991).

The dispersal of *Ae. albopictus* into M.X. is slightly more convoluted. Herein we will discuss the first reports of *Ae. albopictus* in and around M.X. With the emergence of *Ae. albopictus* in the Southern U.S., additional surveillance for the species was conducted, and *Ae. albopictus* was first detected in M.X. in 1988 in Matamoros, Tamaulipas, a city near the Texas border (CDC 1989). Later, in 1993, *Ae. albopictus* was found in three separate cities of Coahila (Ibáñez-Bernal, Sergio; Martínez-Campos 1994, Rodriguez Tovar and Ortega Martinez 1994), another state that is along the southern Texas border. In 1995, a preliminary study in Guatemala revealed that *Ae. albopictus* was established in Puerto Barrios, a major port along the Caribbean Sea, but not in other major entry points into the country (Ogata and Samayoa 1996). The next reports of *Ae. albopictus* occurred in 1997 when the species was found in Allende City, Nuevo

León (Orta-Pesina et al. 2001) and Martínez de la Torre, Veracruz (Flisser et al. 2002). Despite the previous establishment of *Ae. albopictus* in Guatemala and several Northeastern states of M.X. in the early 1990s, it was not until 2002 when *Ae. albopictus* was first reported in our study site, Tapachula, Chiapas (Casas-Martinez and Torres-Estrada 2003). The next reports of *Ae. albopictus* were in 2009 when it was found in Central M.X. in the State of Morelos (Villegas-Trejo et al. 2010), and also near the Guatemala border in Belize (Ortega-morales et al. 2010). Subsequent reports of *Aedes albopictus* in M.X. occurred in Quintana Roo in 2011 (Salomón-Grajales et al. 2012), San Luis Potosi in 2012 and 2013 (Ortega-Morales and Rodríguez 2016) and in Sinaloa in 2014 (Torres-Avendaño et al. 2015). Evidence from a population genetics study suggested that the *Ae. albopictus* in M.X. originated from the U.S. and Central and South America by land (Pech-May et al. 2016).

**History of Vector Control in the Americas Prior to Arrival of *Aedes albopictus***

Upon reviewing the geographical dispersal of the two *Aedes* mosquitoes into the New World, it is important to consider how these two closely related species of mosquitoes may have endured different exposure to insecticide pressure over the course of time in the Americas. We will first consider the history of *Ae. aegypti* in the Americas. At the beginning of the 1900s, Carlos J. Finlay first surmised that *Ae. aegypti* mosquitoes were transmitting yellow fever virus (YFV) to humans (Lee 1905). It was not until the end of the 19$^{th}$ century when Finlay's theory was finally accepted by Walter Reed and the Yellow Fever Commission (Finlay 1903, Lee 1905, Schliessmann 1964, Slosek 1986). Shortly after it was confirmed that *Ae. aegypti* was the primary vector of YFV, William C. Gorgas initiated control efforts in Havana, Cuba in 1901, and later in 1904 in the Panama Canal region, targeting *Ae. aegypti* mosquitoes and demonstrating

that yellow fever could be eradicated in these areas (Gorgas 1915, Schliessmann 1964, Slosek 1986).

After demonstrating that *Ae. aegypti* eradication was possible in these areas, several Latin American countries began implementing their own efforts to eradicate *Ae. aegypti* mosquitoes (Slosek 1986). While these efforts seemed to be effective at the time, a resurgence of yellow fever in Brazil and a new understanding of the jungle cycle of yellow fever resulted in many countries deprioritizing the eradication efforts, since it seemed futile to combat the disease in rural and natural areas (Slosek 1986). This led to a reestablishment of the mosquito in many of these previously treated areas (Slosek 1986). It was not until the 1940s when eradication efforts began again, after Brazil had been experiencing recurring epidemics of yellow fever and approached the Pan American Health Association (PAHO) to implement these efforts in the Americas (Slosek 1986). Many Latin American countries completed these initial eradication efforts between 1958 and 1965 (Slosek 1986), however, the U.S. did not initiate efforts until 1964 (Schliessmann 1967), and ended up prematurely dropping out of the program in 1969, supposedly due to lack of funds (Slosek 1986).

While the efforts of the PAHO eradication program were effective at first and eliminated *Ae. aegypti* in many of the participating countries (Schliessmann 1967), the program as a whole failed due to lack of complete and continued compliance among all the countries with *Ae. aegypti* populations. Perhaps even with full involvement of all affected countries, the program would have still failed due to lack of foresight on selecting for insecticide resistance at the time. It also would have been difficult to anticipate the development of cross-resistance between insecticides, since it was not until the 1970s when patterns of cross-resistance in the U.S. were first reported in the housefly (Farnham 1971) and the mosquito *Culex pipiens quinquefasciatus*

(Priester and Georghiou 1978). While the abandonment of the eradication program allowed for the resurgence of *Ae. aegypti* in many regions, it is possible that other factors also likely impacted the level of insecticide resistance we currently observe today in many *Ae. aegypti* populations.

For example, at the beginning of the U.S. involvement in the eradication program in the summer of 1964, treated areas included parts of Puerto Rico and Florida, the Virgin Islands, and areas along the U.S./Mexican border (Schliessmann 1967), which seemed to align with the regions in the U.S. where *Ae. aegypti* populations could survive year round (Slosek 1986). Perhaps the lack of targeting other surrounding southern states could have contributed to resistance if *Ae. aegypti* on the outskirts of these treated areas were being exposed to sublethal doses of insecticides. However, the types of insecticides used were probably bigger components to this unforeseen problem. Schliessmann mentioned that during the initial U.S. efforts in the eradication program, Dichlorodiphenyltrichloroethane (DDT, an organochlorine) was the primary insecticide used, while malathion (an organophosphate) was used in cases where resistance to DDT was occurring (1967). While it was notable that an alternative insecticide was used for DDT-resistant populations, it is unclear as to how carefully this resistance was monitored and managed. It is also difficult to say how and which insecticides were used in other countries in the PAHO eradication program. Of course, even if these issues would have been carefully coordinated and planned out among the American countries, it is unlikely they still could have anticipated the cross-resistance that would later be recognized between DDT and pyrethroids.

Regardless of whether this severity of insecticide resistance could have been avoided in the Americas, it is interesting that *Ae. aegypti* was the prime target of the mosquito eradication

efforts, and the failure of the program seems to have resulted in heavy selection for insecticide resistance in this species. However, *Ae. albopictus* was not introduced to the New World until the mid- 1980s, which means that New World *Ae. albopictus* have not been historically exposed to insecticides in the same way as New World *Ae. aegypti*.

Yet we still must consider the possibility that *Ae. albopictus* could have developed resistance to insecticides prior to their introduction to the New World. According to a report from the World Health Organization (WHO), countries in the South-East Asia region were targeting and controlling malaria vectors (*Anopheles* spp.) primarily with DDT, hexachlorocyclohexane (HCH, another organochlorine), and malathion as part of the malaria eradication program that began in the 1950s (WHO Expert Committee on Insecticides & World Health Organization 1986), and so presumably, *Aedes* mosquitoes in this region were also being exposed to these insecticides. In this same report, it was mentioned in certain parts of the world, *Ae. aegypti* had already developed resistance to DDT, organophosphates, carbamates and pyrethroids, while *Ae. albopictus* was only found to be resistant to DDT and organophosphates at the time (WHO Expert Committee on Insecticides & World Health Organization 1986). Therefore, it appears that *Ae. albopictus* had not developed resistance to pyrethroids prior to the species being introduced into the Americas.

**Biology, Ecology and Behavior of *Aedes albopictus***

Holometabolous insects, such as mosquitoes, develop through multiple morphologically distinct life stages including the egg, larval, pupal and adult stages. *Aedes albopictus* mosquitoes are a multivoltine species, which means that they can produce 5-17 generations in a year (Gatt et al. 2009). The adult female mosquitoes lay eggs in containers that hold water, which can be

naturally or artificially derived, such as tree holes, rock pools, coconut husks, cans, tires, flower pots, etc. (Estrada-Franco and Craig 1995). The eggs are fertilized as they are laid (assuming the female was previously inseminated by a male) but are not viable until embryogenesis has occurred. Embryogenesis usually takes about 2 to 4 days, but can take up to even 6 or 7 days depending on the temperature, humidity and whether the *Ae. albopictus* are American or Asian lineages (Estrada-Franco and Craig 1995).

After embryogenesis has occurred, eggs are stimulated to hatch by the presence of water and decreased oxygen levels in the water. A decline in dissolved oxygen levels is a signal of microbial activity and indicates that the environmental conditions are suitable for the larv*Ae.* When the eggs are stimulated, fully formed 1$^{st}$ instar larvae will hatch out, and then successively molt into 2$^{nd}$, 3$^{rd}$, and 4$^{th}$ instar larvae over a period of about 6 to 13 days, depending on the temperature, food availability, larval density, and sex of the larvae (Estrada-Franco and Craig 1995). Under field conditions, *Ae. albopictus* larvae will feed on microorganisms and organic detritus present in the water (Foster and Walker 2002). After the 4$^{th}$ instar, the larvae molt into the pupal stage, which usually lasts about 2 days (but can be longer at lower temperatures), after which the adult emerges out from the pupal case and onto the water surface, with the males typically emerging earlier than the females (Estrada-Franco and Craig 1995, Foster and Walker 2002).

After emergence, adults of both sexes will seek sugar sources such as plant nectar or rotting fruit to meet energetic demands. To obtain protein for egg production, females will then generally blood-feed after about 2-3 days post eclosion (d.p.e.) (Hawley 1988) but have been observed to take a blood meal within the first 24 hours of eclosion (Hien 1976). Adult *Ae. albopictus* females have been shown to live from about 1 to 2 months under laboratory

conditions (males typically do not live as long), and in some cases up to 6 months (Hawley 1988) depending on humidity, diet, and temperature (Estrada-Franco and Craig 1995). It is more difficult to estimate the longevity of *Ae. albopictus* in the field, but one mark-release-recapture study found that *Ae. albopictus* females collected from a tire scrap yard in Missouri survived 8.2 days on average, while males only survived 3.9 days (Niebylski and Craig 1994). In general, females are roughly expected to live about 3 to 11 days on average (Bara et al. 2015), but have been found to be able to survive up to several weeks in the wild (Hawley 1988). Depending on their longevity, they can take multiple bloodmeals in a lifetime, thereby increasing their chances of transmitting pathogens (Estrada-Franco and Craig 1995).

While both *Ae. aegypti* and *Ae. albopictus* can enter states of quiescence in order to survive unfavorable environmental conditions, *Ae. albopictus* mosquitoes are better able to survive in colder environments due to the biological trait known as diapause (Diniz et al. 2017). Diapause, which has also been observed in other mosquito and insect species, is an important factor in the biology of *Ae. albopictus* that has allowed the species to expand its distribution to areas where *Ae. aegypti* cannot. Diapause is a genetic trait observed in *Ae. albopictus* that live in temperate regions, which protects the eggs during cold winter seasons and prevents hatching during this time, and is stimulated by photoperiod and temperature (Estrada-Franco and Craig 1995, Diniz et al. 2017). Hawley et al. (1987) demonstrated that *Ae. albopictus* eggs from temperate regions were hardier compared to tropical-derived *Ae. aegypti* and *Ae. albopictus* eggs. After exposing the mosquito eggs to -10°C temperatures for 24 hours, they found that the temperate *Ae. albopictus* egg mortality was no greater than 22%, while there was nearly 100% mortality in the eggs from tropical mosquitoes (Hawley et al. 1987). These adaptive traits to cold

weather have clearly given *Ae. albopictus* an advantage over other mosquitoes in temperate regions where temperatures can be greatly reduced during parts of the year.

Another biological trait that has been observed in *Ae. albopictus* from this study, as well as previous studies, is autogeny (Bat-Miriam and Craig 1966, Cui 1982, Chambers and Klowden 1994, Mori et al. 2008). Autogenous mosquitoes can produce mature eggs without a bloodmeal during their first gonotrophic cycle. Autogeny is increased in females that obtained adequate larval nutrition, can obtain sugar sources after emerging, and have had mating opportunities (Mori et al. 2008). This trait can be a favorable advantage to *Ae. albopictus* when food is abundant for larvae and when hosts are scarce and/or defensive against adult mosquitoes (Mori et al. 2008) and can be ecologically impactful in helping to maintain the species when bloodmeals are difficult to obtain (Estrada-Franco and Craig 1995).

*Aedes albopictus* is a fairly opportunistic feeder that is willing to feed on a wide variety of hosts, such as dogs, rabbits, squirrels, humans, deer, birds (Savage et al. 1993) and even frogs and turtles (Niebylski et al. 1994, Richards et al. 2006) depending on host availability. The feeding behavior of this invasive species is suspected to have partially contributed to its rapid dispersal across the U.S. (Richards et al. 2006) and gives yet another survival advantage to *Ae. albopictus* in various habitats with differing host-species availability. However, there have been several studies that have shown *Ae. albopictus* can have a preference for feeding on humans (Delatte et al. 2010, Kamgang et al. 2012). In general, *Ae. albopictus* is considered an exophilic, daytime biter, but this can vary depending on the locality, habitat, season, and availability of hosts (Paupy et al. 2009). While *Ae. aegypti* is also a daytime biter, it is considered to be an endophilic biter and has a high preference for humans (anthropophilic) (Gubler 1998).

## Vector Competence and Capacity of *Aedes albopictus*

An arthropod's ability to become infected with, replicate, disseminate and transmit a virus is described by the term vector competence (Monath 1988, Kramer and Ciota 2015), which can vary depending on the species of arthropod and virus. While vector competence is important to consider when evaluating the potential emergence and spread of arboviruses (viruses transmitted by arthropods), we must also consider the population density, biting behavior, and longevity of the arthropod vectors (Kramer and Ciota 2015). Cumulatively, these other factors, along with vector competence, affect the vectorial capacity. Vectorial capacity considers the intrinsic and extrinsic factors that affect how well an arbovirus can be spread by an arthropod under certain conditions.

With regards to the vector competencies of *Ae. aegypti* and *Ae. albopictus*, they are both capable of transmitting Zika virus (ZIKV), dengue virus (DENV), chikungunya virus (CHIKV) and yellow fever virus (YFV) (Weaver and Reisen 2010, Kauffman and Kramer 2017, Garcia-Luna et al. 2018, Amraoui et al. 2019). Additionally, *Ae. albopictus* is a competent vector for at least 23 other arboviruses (Paupy et al. 2009). While *Ae. albopictus* is generally a less competent vector for these five major arboviruses compared to *Ae. aegypti*, that does not necessarily mean that we should be less concerned or worried about *Ae. albopictus* regarding its ability to spread diseases or potentially cause outbreaks. For example, *Ae. albopictus* was found to be the vector responsible for the CHIKV epidemic of 2005-2006 on Reunion island, rather than *Ae. aegypti*, which is usually implicated as the primary vector (Reiter et al. 2006). It was later found that mutations in the envelope glycoprotein genes (E1 and E2) of the CHIKV strain that was associated with the Reunion epidemic had enhanced the ability of the virus to be disseminated and transmitted by *Ae. albopictus* (Tsetsarkin et al. 2007, Tsetsarkin and Weaver 2011). This was

also demonstrated under laboratory conditions, which showed the Reunion CHIKV strain was more efficiently transmitted than an Asian-derived CHIKV strain by *Ae. albopictus* collected from the U.S. (Sanchez-Vargas et al. 2019). Results from this study suggested that if the Reunion strain of CHIKV were introduced into susceptible human populations of the western hemisphere, the risk of its transmission would be increased by both *Aedes* species (Sanchez-Vargas et al. 2019).

As with CHIKV, *Ae. aegypti* is generally described as the primary vector of DENV (Chan et al. 1971). However, previous reports and observations have shown that *Ae. albopictus* can also have significant impacts on the emergence and maintenance of DENV in the field. In instances when *Ae. aegypti* or other vectors are absent or scarce, *Ae. albopictus* has been clearly implicated as the primary vector of DENV (Gratz 2004). In 1943 *Ae. albopictus*, while still regarded as a less efficient vector of DENV, was considered to be the primary vector of the dengue epidemic in Hawaii because it was present in higher abundance than *Ae. aegypti* in urban areas and was also found in areas where DENV was being transmitted even though *Ae. aegypti* was completely absent (Gilbertson 1945). During another dengue outbreak in Hawaii in 2001, *Ae. albopictus* was again the responsible vector as indicated by the sparse inhabitance of *Ae. aegypti* on the islands (Effler et al. 2005). In at least two other instances prior to this, in Japan and Seychelles, *Ae. albopictus* was found to be the only dengue vector present, and was ultimately responsible for the epidemics (Sabin 1952, Metselaar et al. 1980). In certain areas of China, *Ae. albopictus* is considered the primary vector of DENV, due to the absence or low occurrence of *Ae. aegypti* in these areas, and due to the high frequency of virus isolation from *Ae. albopictus* in these regions (Gratz 2004). However, even in areas where *Ae. aegypti* is the primary vector of DENV, *Ae. albopictus* can still play a role in the maintenance of the virus.

Vazeille et al. (2003) mentions that while their study found *Ae. aegypti* to be significantly more receptive to oral infection with DENV serotype 2, *Ae. albopictus* could still be contributing to the maintenance of the virus in nature where susceptible human and non-human hosts are not available. This is because the latter species is more efficient at vertically transmitting the virus by transovarial transmission from females to their offspring, and *Ae. albopictus* males are capable of infecting females during copulation (Vazeille et al. 2003), allowing maintenance of the virus within the mosquito populations. When we also consider the autogenous capability of *Ae. albopictus*, it seems feasible for the species to maintain the virus in nature through vertical transmission when hosts are absent.

Recall that vectorial capacity is more than just vector competence. Under certain conditions, such as when the extrinsic incubation period is reduced, the vector population density is large, or when feeding behavior of the vector increases, an outbreak can persist and expand even if the vector has a low competence for the pathogen (Kramer and Ciota 2015). Some studies have argued that the vector potential of *Ae. albopictus* is lowered due to its opportunistic feeding behaviors, since it will feed on various mammals and sometimes reptiles and amphibians (Richards et al. 2006), but others have argued that this could potentially make *Ae. albopictus* a more likely candidate as a bridge vector for zoonotic diseases (Vazeille et al. 2003, Gratz 2004, Delatte et al. 2010). Even still, other studies that have shown *Ae. albopictus* can have a preference for biting humans in some situations, which they argue could increase its potential to transmit viruses between humans under these settings (Kamgang et al. 2012). Therefore, while *Ae. aegypti* tends to be a more competent vector for arboviruses, we still must acknowledge that under certain conditions *Ae. albopictus* can be responsible for arboviral outbreaks and maintenance of the viruses in nature.

**Impacts of Disease Caused by *Aedes* Mosquitoes**

Given the information discussed in the previous section, it is of vital importance to control both species of *Aedes* mosquitoes to reduce the spread of arboviral diseases and the severe impacts they can have on public health. Some symptoms of these diseases can be fairly mild and similar in certain cases; such as with ZIKV, DENV and CHIKV, individuals typically display common symptoms such as fever, rash, headache, myalgia and arthralgia (Lei et al. 2001, Simon et al. 2007, Petersen et al. 2016). However, oftentimes people do not even realize they have these diseases because they assume the symptoms are caused by something else, like the common cold or flu, or in many instances individuals can be asymptomatic, which means that the number of cases for these diseases can often be underreported.

However, each of these diseases can also present more severe symptoms in other cases. During the recent and large ZIKV outbreaks that occurred in 2015 and 2016, it was found that the virus can cause microcephaly and other birth defects in infants if a mother is infected during pregnancy, and it has also been shown to cause Guillain-Barré syndrome, which causes an individual's immune system to attack their nerve cells (WHO 2017). There are also cases in which patients who are infected with dengue can progress into more severe forms of the disease; dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS), which can result in internal bleeding, shock and sometimes death (Lei et al. 2001). This is very concerning for pregnant women, infants, and people previously infected with DENV because they are more at risk of experiencing severe dengue infections (CDC 2019a). While CHIKV infection does not often lead to death; it can be severely debilitating due to the severe arthralgia some patients experience. Hence the name "chikungunya," which describes the contorted posture of those suffering from the disease (Ross 1956, Simon et al. 2007). However, in some instances CHIKV can cause

mortality, as observed during the chikungunya epidemic of Reunion in 2005-2006, which resulted in at least 260 deaths (Charrel et al. 2007).

As for YFV, the symptoms that result from infection can be more severe than the three previously discussed arboviruses. At initial onset of disease, patients usually experience, fever, chills, headache, conjunctival congestion, myalgia, nausea, and dizziness, followed by a period of remission (Monath 2001). Some people will recover after this stage, while about 15-25% of people progress to a more severe form and experience symptoms such as vomiting, jaundice, renal failure and spontaneous hemorrhaging, and of those who contract the severe form, about 20-50% die (Monath 2001). Given the severe impacts that all of these diseases can have on public health, it is vital for us to control the spread of these arboviruses.

**Arbovirus Disease Prevention Strategies and the Rising Problems with Insecticide Resistance**

Vaccines can be a useful tool to help limit the spread of arboviruses, however only two out of the four, YFV and DENV, currently have vaccines available to prevent infection. The yellow fever vaccine (YF-17D) was first developed in the 1930s (Theiler and Smith 1937). The vaccine is very effective and typically will provide life-long immunity against the virus (Monath 2001). Fortunately, with the development of vector control programs and an effective vaccine, medical impacts and fear of contracting yellow fever have been reduced (Monath 2001). However, despite these advancements, YFV still continues to be a problem in endemic regions as demonstrated by the numerous outbreaks in South America and Africa and the resulting imported cases into other countries from infected travelers (Barrett 2018). Funds for the vaccine are limited and are used to stockpile vaccines for epidemic responses, while preventative

vaccination is prioritized for at-risk endemic countries when vaccines are left over from the outbreak stockpile are not used within a certain timeframe (Yen et al. 2015). Unfortunately, depletion of the vaccine seems to be a common occurrence. During the outbreaks of 2016 in Africa and Brazil, world vaccine supplies were depleted multiple times (Barrett 2018). Even now, the YF-17D vaccine is currently unavailable, and an alternative vaccine, Stamaril, is in limited supply at certain clinics in the U.S. (https://wwwnc.cdc.gov/travel/news-announcements/yellow-fever-vaccine-access). While cases that are imported into non-endemic countries can easily be prevented by vaccination, and even though YFV immunization is required by International Health Regulations to enter yellow fever endemic countries, many countries do not strictly enforce this (Monath 2001).

The vaccine against dengue (CYD-TDV or Dengvaxia) has only recently been licensed in M.X. in 2015. Since then it has been licensed in other dengue-endemic countries, but is not yet widely available, and other vaccine candidates against dengue are still undergoing clinical development (Vannice et al. 2016). Due to the limitations of these vaccines and the lack of vaccines against other harmful arboviruses, the best way to currently control the spread of these diseases is by targeting and killing the mosquito vector.

Mosquito control can be targeted towards the larval or adult stages using a variety of methodologies. Targeting the immature stages of mosquitoes is often an effective way to reduce populations because unlike adults, larvae are relatively immobilized and confined to the breeding sites from which they hatched (Floore 2006). Reduction of mosquito breeding sites is one effective and potentially long-term solution for controlling mosquito populations. When specifically dealing with container-breeding species like *Ae. albopictus* and *Ae. aegypti*, residents are advised to dispose of or cover containers that can hold water, such as plastic containers and

tires, and to prevent water build up in structures when possible, such as cleaning gutters and changing out water from bird baths regularly (Floore 2006). Another option is to treat breeding sites with larvicides such as insect growth regulators (IGRs, e.g. methoprene), microbial insecticides (e.g. *Bacillus thuringiensis* var. *israelensis* [*Bti*]), and organophosphates (e.g. temephos and malathion) (Floore 2006). Unfortunately, due to development of resistance, these compounds are not always effective. Biological control methods are an additional way to kill mosquito larv*Ae.* Larvivorous predators, such as *Gambusia* spp. fish, *Toxorhynchites* spp. larvae, and certain species of copepods have been used as biological control methods against mosquitoes (Benelli et al. 2016). However, certain complications can arise with these methods, such as the potential to disrupt the aquatic ecosystem by introducing new invasive species into certain environments, as well as the opposite effect in which the larvivores do not become established in the targeted water source and therefore do not provide continued control of the mosquito larvae (Benelli et al. 2016). Additionally, larval control can be difficult, regardless of the methodology, when specifically trying to target *Aedes* spp. because oftentimes their breeding sites are cryptic and not always easy to locate (Unlu et al. 2013, Valença et al. 2013, Vijayakumar et al. 2014).

Alternatively, vector control methods can be targeted at the adult mosquitoes. Luring and killing mosquitoes can help reduce mosquito populations, using methods such as attractive-toxic sugar baits, lethal ovitraps, sticky traps or host-seeking traps (Faraji and Unlu 2016). Auto-dissemination stations are another type of attract-and-kill technology in which mosquitoes are lured to a station and then contaminate themselves with pyriproxyfen, another IGR, and then disperse it to larval breeding habitats (Faraji and Unlu 2016). This method can be useful when attempting to control *Aedes* spp. because the mosquitoes disperse the IGR to cryptic breeding containers that would otherwise be difficult for vector control personnel to locate and treat

(Faraji and Unlu 2016). However, the efficacy of these methods depend on how well the mosquitoes are attracted to the device, if the bait or device outcompete the food and breeding sources that are naturally present in the environment, and the number of devices needed to provide area-wide control (Faraji and Unlu 2016).

Limitations of these previously described methods, along with technological advancements, has led to the development of novel approaches for mosquito control, specifically the release of modified mosquitoes into wild populations. While this may seem counterintuitive, the goals of these methods are either to suppress or modify wild mosquito populations, and ultimately reduce the transmission of arboviruses. Suppression of populations can be achieved through sterile insect technique (SIT) or Release of Insects carrying a Dominant Lethal (RIDL). SIT is performed by sterilizing males with radiation or chemical treatment, and then releasing them to breed with wild females, which then cannot produce offspring due to the dominant lethal mutations present in the sperm of the treated males (Benelli et al. 2016, Flores and O'Neill 2018). The RIDL technique, pioneered by Oxitec, suppresses populations by creating transgenic males that have a lethal gene that can be repressed when mosquitoes are given tetracycline in the lab, but then when they are released into the wild, males pass this transgene onto their progeny, which end up dying in the late larval stage due to the absence of tetracycline in the environment (Flores and O'Neill 2018). The benefit of these two methods are that they do not require the release of females into the wild, making it easier to get public approval for implementation. However, these methods require releasing large numbers of mosquitoes and continuous reapplications in order to suppress populations effectively (Flores and O'Neill 2018).

Another method that can suppress populations involves releasing male mosquitoes infected with *Wolbachia pipientis* (an endosymbiotic bacterium, from here on referred to as

*Wolbachia*), which breed with uninfected wild females, resulting in embryonic death due to cytoplasmic incompatibility induced by the *Wolbachia* (Flores and O'Neill 2018, Shaw and Catteruccia 2019). This method acts similarly to SIT and is also referred to as incompatible insect technique (IIT). Alternatively, both males and females infected with *Wolbachia* can be released to make wild populations refractory to infection by certain viruses, such as DENV and ZIKV (Benelli et al. 2016, Flores and O'Neill 2018). Compared to SIT and IIT, fewer mosquitoes need to be released into the wild and typically do not require multiple deployments in order to drive *Wolbachia* infections into the population, because females can reproduce successfully with infected and non-infected males (Flores and O'Neill 2018).

Newly emerging techniques utilizing gene drive mechanisms are becoming available and can potentially be used to modify or suppress mosquito populations. Researchers are now investigating the potential use of CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats-associated protein 9) in vector control. The system is comprised of a guide RNA that recognizes the insertion site sequence and directs where the Cas9 endonuclease (encoded by the transgene) makes a cut on the non-transgenic chromosome, and upon homology directed repair of the cleaved chromosome, the transgene is used as a template, resulting in a homozygote that will pass the transgene on to all of the offspring (Flores and O'Neill 2018, Shaw and Catteruccia 2019). This gene drive system in mosquitoes is still in the preliminary stages of testing and promising results have at least been observed in two *Anopheles* species (Gantz et al. 2015, Hammond et al. 2016, Shaw and Catteruccia 2019), however this system has yet to be tested in the field (Flores and O'Neill 2018). The CRISPR-Cas9 system can potentially be used to modify or suppress mosquito populations by introducing transgenes that reduce vector competence or reproductive capabilities. Another benefit of the CRISPR-Cas9 system is that

very few mosquitoes would need to be released for the transgene to be driven into the population, however, this also causes concern among the community due to the unforeseen risks and potential problems that could arise from an uncontrolled gene drive (Flores and O'Neill 2018).

While technology and research have led to a variety of methods to suppress mosquito populations and reduce the transmission of arboviruses, effective vector control is still heavily reliant on insecticide applications. Insecticides can be implemented in a variety of ways to control adults, such as ultra-low-volume (ULV) aerial or ground sprays, indoor residual spraying (IRS), and insecticide-treated materials such as bed nets and clothing (Bonds 2012, Roizid et al. 2018). However, there are ongoing challenges and concerns with insecticide use, including the negative effects that can possibly occur to the environment and non-target insect species, effects on human health from insecticidal exposure, and the development of resistance to active ingredients (Benelli et al. 2016). Fortunately there are insecticide resistant management (IRM) strategies, such as continued monitoring of resistance, limiting the use of insecticides and rotating insecticides of different chemical classes that can help maintain the effectiveness of insecticides (WHO 2012). While insecticides are an important tool that is still needed to control mosquito vectors, they are not effective enough on their own, as is the case with any of the previously discussed control methods. Ultimately, the most effective way to control mosquitoes is through integrated mosquito management (IMM) practices, which includes epidemiological and entomological surveys, larval and adult control through various methods, as well as engagement and education of the community (Faraji and Unlu 2016, Roizid et al. 2018).

**Pyrethroid Insecticides: What, Why and How?**

While there are many different types of insecticides used to control mosquitoes and other insect pests, and while resistance has been observed in every insecticidal chemical class (Brogdon and McAllister 1998), herein our discussion will primarily focus on permethrin, since it was the insecticide of choice for this study. We chose to use permethrin because it is a commonly used insecticide worldwide and resistance to this insecticide has become evident in many mosquito species. Although resistance is becoming more prominent, permethrin is still often chosen as a means for control because of its rapid knockdown effect and its low toxicity to humans and other mammals (WHO/CDC/WHOPES/GCDPP 2005).

Permethrin is a synthetic pyrethroid insecticide that was derived from the pyrethrum extract found in *Chrysanthemum cinerariaefolium* and *C. roseum* flowers (Housset and Dickmann 2009, Yu 2015). The original use of pyrethrum was supposedly derived in Asia, possibly around 2000 years ago, in which the flower heads were dried and ground into an insecticidal powder, known as "Persian Insect Powder," and was sold by merchants to people throughout Russia and western Europe (Howard 1910, Housset and Dickmann 2009). The synthetic version of permethrin, along with cypermethrin and deltamethrin, were not produced until 1972 (Housset and Dickmann 2009). Currently, synthetic permethrin and other pyrethroids are generally preferred over the natural extract for use in public health and agriculture because they are cheaper to produce, have higher photostability, and have higher insecticidal activity (Housset and Dickmann 2009, Yu 2015). Pyrethroids are further categorized into type I and type II classes, with permethrin falling under the former category. Type I and II pyrethroids have similar chemical structures (Figure 1.1), but type II pyrethroids typically also have a cyano group (Soderlund and Bloomquist 1989, Yu 2015). They are also classified separately based on their

neuronal effects on the American cockroach: type I pyrethroids cause neurons to repeatedly discharge and have a rapid effect resulting in whole-body tremors, while type II pyrethroids cause the nerve membrane to slowly depolarize and decreases the electrical excitability resulting in sinuous writhing (Soderlund and Bloomquist 1989, Bloomquist 1996, Yu 2015).

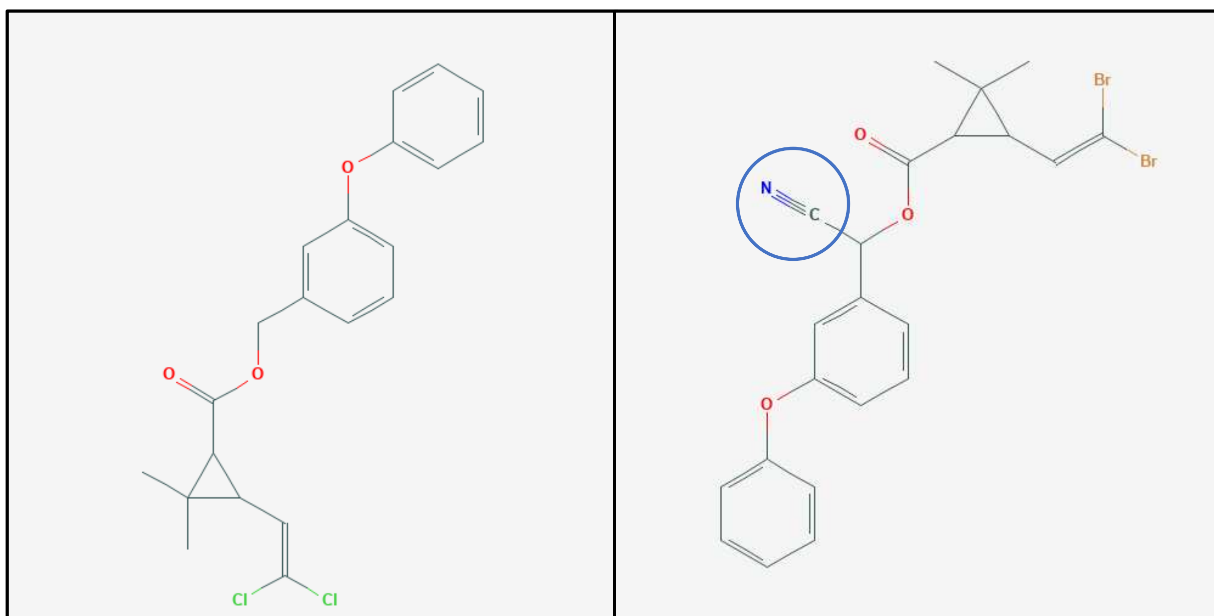


Figure 1.1: Chemical structures of permethrin (left), a type I pyrethroid, and deltamethrin (right), a type II pyrethroid. The cyano group of deltamethrin is circled in blue. Figures modified from https://pubchem.ncbi.nlm.nih.gov/

Both types of pyrethroids, along with DDT and its analogs, cause these toxic effects by binding to the voltage-gated sodium channels (VGSC) in the neurons of the insect, which forces these channels to remain open, thereby disrupting proper delivery of nerve impulses (Yu 2015). The VGSC is a large transmembrane protein of nerve cells that is composed of a single polypeptide chain α-subunit containing four homologous domains (DI – DIV), each consisting of six α-helical segments (S1 – S6), that fold together to create the selective ion pore (Figure 1.2) (Catterall 2000, Namadurai et al. 2015). In insects, the α-subunits of VGSCs are encoded by the *para* gene (in *Drosophila melanogaster*) and its orthologs (Soderlund 2010). Under normal

conditions, VGSCs (accompanied by potassium channels), facilitate the transmission of signals through nerve cells (Yu 2015). When the membrane potential of the neuron is at rest, the sodium channel is in the closed position (inactivated state) and as a nerve impulse travels along the axon, the membrane becomes permeable to sodium ions as the sodium channel opens (activated state) and the ions flow into the axon, depolarizing the membrane (Vais et al. 2001, Hemingway et al. 2004, Yu 2015). After about 1 ms, the conformation of the sodium channel changes, leaving it in an open but blocked state, which prevents sodium ions from entering the cell, and when the membrane is repolarized and returns to its resting state, the sodium channel closes (Vais et al. 2001, Hemingway et al. 2004, Yu 2015). Pyrethroid insecticides bind to the VGSCs and delay the closing of the sodium channel, prolonging the inactivation of the channel, resulting in repetitive firing of nerve impulses and excessive neuroexcitation (Vais et al. 2001, Yu 2015).

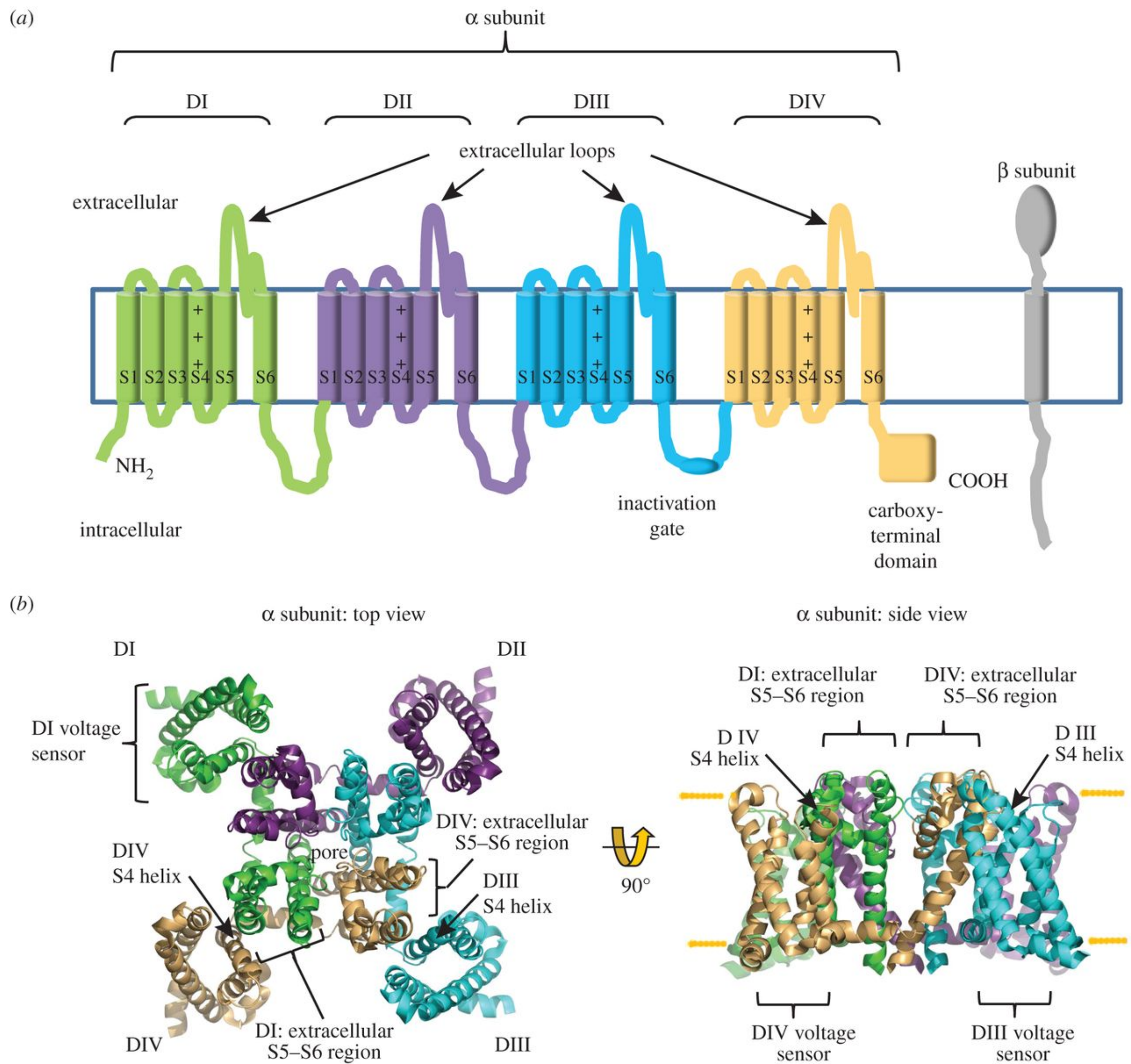


Figure 1.2: Structural diagram of the voltage-gated sodium ion channel (VGSC). (a) Illustration of the α- subunit of the VGSC, showing the four domains within the α-subunit and the six transmembrane helices (segments) within each domain, and the inactivation gate. Note that the β- subunit is also shown, but is associated with VGSCs in vertebrates, not insects. (b) Top view (left) and side view (right) of a 3-dimensional representation of the VGSC to model the physical structure of the VGSC protein in the cell membrane, and how the segments of each of the four domains conform to create the pore through which the sodium ions enter into the nerve cell. Figure is from Namadurai S., Yereddi N.R., Cusdin F.S., Huang C.L.-H., Chirgadze D.Y., Jackson A.P. 2015. "A new look at sodium channel β subunits." *Open Biol*. 5: 140192. http://dx.doi.org/10.1098/rsob.140192 (Namadurai et al. 2015).

**Insecticide Resistance Mechanisms**

For this study we focused on investigating target site insensitivity and detoxification enzymes as possible mechanisms for resistance in our *Ae. albopictus*. When considering DDT and pyrethroid insecticides, target site insensitivity occurs when there are one or more point mutations in the VGSC gene which result in knockdown resistance (*kdr*) to the insecticide (Yu 2015). These mutations change the shape of the VGSC, lowering the binding affinity of the insecticide, which prevents it from keeping the channel open, so the nerve cells are able to function more normally (Brogdon and McAllister 1998, Yu 2015). Specific mutations of the VGSC are covered in detail in Chapter 3.

The other mechanism of resistance that we investigated in *Ae. albopictus* is metabolic resistance, which occurs when the amount or activity of detoxification enzymes are enhanced or modified, which then help break down insecticides and prevent them from attaching to the target site (Brogdon and McAllister 1998). There are three major groups of enzymes that are associated with metabolic resistance to insecticides: cytochrome P450 monooxygenases/mixed function oxidases, esterases, and glutathione S-transferases (GSTs) (Yu 2015). Refer to Chapter 3 for more detailed information about these enzymes and their specific roles in conferring insecticide resistance.

While the focus of this study was to investigate target site mutations and detoxification enzymes associated with resistance, it is still worth mentioning that there are two other types of resistance mechanisms that are observed in insects. The first is behavioral resistance, which is when an insect avoids contact or ingestion of an insecticide dose that would have otherwise been lethal (Yu 2015), but the mechanisms behind this are not very well understood (Chareonviriyaphap 2012). The other type of resistance occurs due to modifications of the

cuticle, which results in reduced penetration of the insecticide (Yu 2015). The two proposed mechanisms for this type of resistance are thickening of the cuticle and alterations of the cuticle components (Balabanidou et al. 2018). It is possible that both behavioral and cuticle mechanisms could also be involved in *Ae. albopictus* resistance, but we were not able to evaluate these mechanisms in this study.

**Aims and Hypotheses of this Study**

The Specific Aims of this study are:

1. Investigate the permethrin resistance status in *Aedes albopictus* populations
    1.1. Test permethrin susceptibility of *Ae. albopictus* from Tapachula, Chiapas, M.X. and Weslaco, Texas, U.S.
    1.2. Artificially select a permethrin-resistant strain of *Ae. albopictus* from the M.X. collection sites.
2. Investigate mechanisms of resistance previously observed in *Aedes* spp. mosquitoes
    2.1. Search and screen for *kdr* mutations at the 1534 site of the VGSC in M.X. *Ae. albopictus* strains.
    2.2. Compare metabolic enzyme activity of M.X. *Ae. albopictus* strains with a known susceptible strain.

At the start of this project, we hypothesized that *Ae. albopictus* from our collection sites would have high levels of resistance to permethrin. We hypothesized this because permethrin (and other pyrethroids) are commonly used to control mosquitoes, and due to the heavy use of these insecticides, mosquitoes have become increasingly resistant to their lethal effects, as has

been observed in *Ae. aegypti* populations (Pereira da-Cunha et al. 2005, Harris et al. 2010, Marcombe et al. 2012, Aponte et al. 2013, Flores et al. 2013). However, the results of this study have not supported our initial hypothesis.

We did not observe high levels of resistance to permethrin in any of our *Ae. albopictus* mosquitoes and did not find the presence of *kdr* mutations in any of the individual mosquitoes that were screened. However, these were peculiar findings due to the presence of high phenotypic resistance and *kdr* mutation frequencies observed in *Ae. aegypti* collected from the same region in M.X. (unpublished data). We now hypothesize that *Ae. albopictus* from our collection sites are less resistant to permethrin than *Ae. aegypti* from the same regions due to differences in selection pressure with insecticides. We suspect that due to the different historical backgrounds of the two species in the Americas, it is possible that *Ae. aegypti* experienced a stronger selection for resistance during the PAHO eradication efforts, and that the late arrival of *Ae. albopictus* in the Americas allowed the species to avoid this intense selection event. An alternative explanation for the contrasting levels of resistance between the two species could also possibly be attributed to their differences in habitat preferences and biting-behavior. Since *Ae. aegypti* tends to be more anthropophilic, and since insecticide applications are more stringently applied to urban areas, it is possible that *Ae. aegypti* has been more directly exposed to and selected with insecticides compared to *Ae. albopictus*. Future studies are needed to confirm these hypotheses.

# CHAPTER 2 – INVESTIGATION OF PERMETHRIN RESISTANCE IN *AEDES ALBOPICTUS* MOSQUITOES

## Introduction

High levels of resistance to permethrin and other pyrethroid insecticides have been reported for *Aedes aegypti* throughout Mexico (M.X.) ( Flores et al. 2009; Flores et al. 2013; Aponte et al. 2013; Kuri-Morales et al. 2018; Lopez-Monroy et al. 2018). Relatively few studies have investigated the resistance status of *Aedes albopictus* populations compared to *Ae. aegypti*. As of January 2020, there have been 252 reports of resistance to 21 active ingredients reported to the Arthropod Resistance Pesticide Database (ARPD) for *Ae. albopictus* (http://www.pesticideresistance.org), compared to *Ae. aegypti*, which has 585 reports of resistance to 35 active ingredients listed on the database. The database is available worldwide, but researchers must apply online and be authorized in order to submit insecticide information on the database. Therefore, this list is not exhaustive and only includes the reports that were submitted directly to ARPD. However, there has yet to be any documentation on the pyrethroid resistance status of *Ae. albopictus* from M.X. Since permethrin is one of the most commonly used insecticides worldwide, one of the goals of this study was to examine whether or not *Ae. albopictus* from M.X. exhibits phenotypic resistance to permethrin.

There is still much to be discovered and understood about the resistance status of *Ae. albopictus* mosquitoes. The overarching goal of this project was to increase knowledge about pyrethroid resistance in *Ae. albopictus* from a region in the world where this has not yet been investigated. It is important to examine and understand the current and future potential of

insecticide resistance in *Ae. albopictus* mosquitoes so that proper control measurements can be implemented to prevent transmission of the pathogens they vector.

As a second aim, we also wanted to select for permethrin resistance in *Ae. albopictus* in the laboratory. Since we were uncertain if we would detect resistance in any of our field populations, we wanted to test if resistance could at least be selected for artificially with regular and continuous exposure to permethrin in the laboratory. Permethrin-resistance selection has previously been achieved with *Ae. aegypti* mosquitoes from Yucatán and Quintana Roo, M.X., where an increase in the $LC_{50}$, $KC_{50}$, and Ile1016 allele frequencies were observed after selecting over five generations (Saavedra-Rodriguez et al. 2012). Another study took moderately permethrin- and deltamethrin-resistant *Ae. albopictus* from Malaysia (Chan, Mustafa, and Zairi 2011) and selected the strain by pressuring larvae with permethrin for seven generations, increasing the RR from 1.5 in the $F_1$ generation, to 99.4 in the $F_8$ generation (Chan and Zairi 2013). Therefore, we hypothesized that if we exposed *Ae. albopictus* mosquitoes to permethrin (via bottle bioassays) and allowed the survivors to reproduce, we would observe an increase in phenotypic resistance among the population after multiple generations.

## Materials and Methods

### Mosquito Collections

Mexican *Ae. albopictus* populations were collected from Tapachula, Chiapas in August and September 2016 (See Figure 2.1). Since there tends to be more insecticide applications within the city of Tapachula, as compared to the rural towns outside of the city (A. Rodriguez, personal communication), we hypothesized that mosquitoes from collection sites within the city would have higher levels of resistance to permethrin compared to the collection sites outside of

the city, and as distance from Tapachula increases, we expected phenotypic resistance to decrease. Unfortunately, there is no reported information available to determine exactly how much and what type of insecticides were being used in our study sites. The most information we could find were the lists of approved insecticides for M.X. for the year prior to (Secretaría de Salud 2015) and the year of (Secretaría de Salud 2016) our sample collections. Permethrin was recommended for use during both years at a 10.87% + concentration for indoor ultra-low volume (ULV) space treatments, among other pyrethroids and classes of insecticides (adulticides and larvicides). There was no way for us to determine the insecticides that were used in Chiapas prior to our sampling data, but based off of these approved lists, we can surmise that permethrin and other pyrethroids were potentially used for mosquito control in the area.

Figure 2.1: Map of *Ae. albopictus* collection sites in and around Tapachula (boxed area), Chiapas, M.X. The collection sites within the city from various neighborhoods were denoted with letters as follows: F (Los Llanes), G (Colonia 5 de Febrero), H (San Agustin), and L (El Porvenir). The sites outside of the city were denoted by the town name from which they were collected. Map by Dr. Farah Vera-Maloof.

Additionally, we were also given *Ae. albopictus* eggs from colleagues in Texas and decided to test these for baseline permethrin resistance to see how they compared to our populations from M.X. *Aedes albopictus* were collected in May/June 2018 from three sites in Weslaco, Texas: West Mile 10 (WM10), Weslaco City Cemetery (WCC) and Estero Llano Grande (ELG). Eggs were obtained from the Gabriel Hamer Lab funded through the Western Gulf Center of Excellence for Vector-Borne Diseases (Figure 2.2). See Appendix for more detailed information on collection sites. The *Ae. albopictus* control strain, ATM-NJ95, generation $F_{12}$, was obtained from Biodefense and Emerging Infections Research Resources

Repository (BEI Resources) as a confirmed insecticide susceptible population (Marcombe et al. 2014). This reference strain was originally established from larvae collected near Keyport, New Jersey in 1995, shortly after *Ae. albopictus* was first detected in the state (Crans et al. 1996).

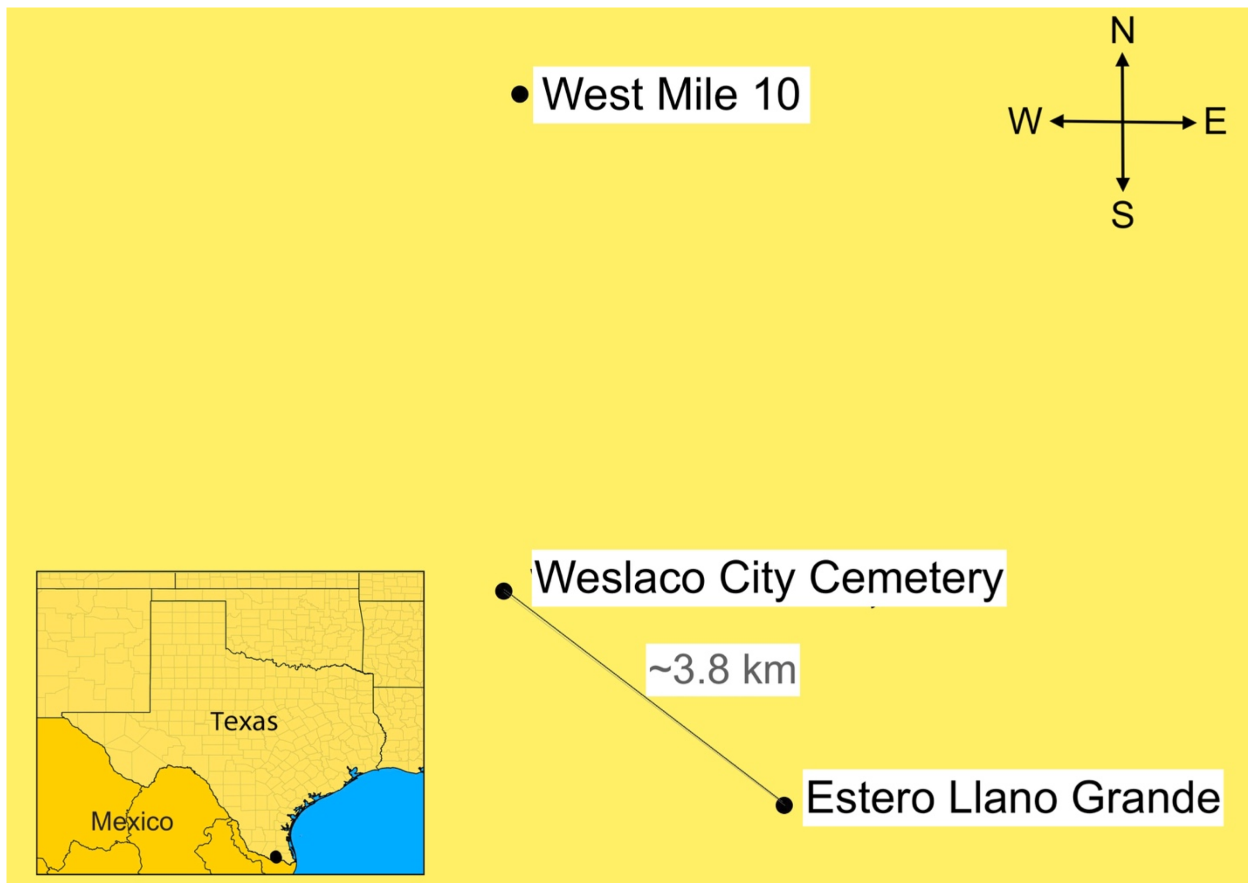


Figure 2.2: Map of *Ae. albopictus* collection sites in Weslaco (denoted by black dot on small inset map), Texas, U.S. The southernmost site, Estero Llano Grande, is approximately 8 km from the U.S./M.X. border. Mosquitoes were provided by Gabriel Hamer Lab. Map by Dr. Farah Vera-Maloof.

Eggs ($F_1$) from M.X. and Texas, and ATM-NJ95 ($F_{12}$) were shipped to Colorado State University (CSU; Fort Collins, Colorado), and were hatched and maintained in the laboratory under insectary conditions (60-80% R.H. and 28-30°C). Eggs were hatched in 4 L clear plastic tubs with approximately 2 L of autoclaved tap water and larvae were fed a 10% liver powder

(MP Biomedicals LLC, Irvine, CA, U.S.) solution (10 g of liver powder + 100 mL of tap water). Pupae were removed from larval tubs and placed in 30 cm x 30 cm x 30 cm cages (BugDorm-1, Mega View Science Co., Ltd., Taichung, Taiwan). Adult mosquitoes were given raisins *ad libitum*. Adults from the initial generations hatched in the lab were identified morphologically as *Ae. albopictus* upon emergence by the presence of a distinct dorsal white stripe down the middle of the thorax (see Figure 2.3). Any *Ae. aegypti* that emerged were removed from the population by aspiration to ensure the strains consisted entirely of *Ae. albopictus* mosquitoes.

Figure 2.3: Female *Aedes albopictus* mosquito, denoted by the white striped pattern of scales on the dorsal thorax. Photo by Ashley Janich.

Bottle Bioassays

Bottle bioassays were modeled after the CDC bottle bioassay protocol (CDC 2013). 250 mg of permethrin (mix of cis and trans isomers) PESTANAL® was purchased from Sigma-Aldrich (St. Louis, Missouri, U.S.) and was mixed with 2.5 mL of 100% acetone to create a 100 mg/mL stock solution (= 100 μg/μL). The 100 μg/μL solution was used to prepare two working solutions, 1.0 and 0.1 μg/μL, which were used to prepare the bottles, and were discarded and remade if they were older than 1 month. All stock solutions of permethrin were stored at 4°C. To prepare bottles, 1 mL of 100% acetone was pipetted into each bottle, and then the appropriate amount of working permethrin solution was pipetted into each bottle to achieve the desired concentration (Refer to Appendix Table A.2.1 for amounts of working permethrin solution used for every concentration). After the permethrin was added, the bottle caps were screwed on tight. The bottle interiors were then coated with the permethrin solution of varied concentrations (0, 0.5, 1, 1.5, 2, and 5 μg/bottle) first by hand (bottles were turned over to ensure every interior part was coated) and were then laterally placed on a Wheaton Bench Top Roller (see Figure 2.4) and were allowed to slowly rotate for 2 min (about 1 rotation every 3.5 sec). After 2 min the caps were loosened and the bottles were left on the roller until the caps completely fell off (this allowed for the solution to disperse evenly throughout the interior of the bottle while the acetone gradually evaporated out, instead of letting the solution settle in one area of the bottle and potentially concentrate the permethrin to that portion of the interior). The bottles were left open overnight in a dark space (e.g. a lab drawer or a cardboard box) to allow any residual acetone to evaporate. Bottles older than 1 week were not used for assays. Each concentration was tested in triplicate for every mosquito strain, using three different bottles (of the same concentration) for each replicate.

Figure 2.4: Picture of Wheaton Bench Top Roller used to prepare bottles for bottle assay. Bottles were laid on their sides on the rollers and were allowed to rotate to evenly distribute permethrin solution within bottle interiors. Photo by Ashley Janich.

Bottle bioassays were first conducted with the $F_3$ generation because earlier generations had too few mosquitoes to test. Adult mosquitoes 3-5 days post eclosion (d.p.e) were aspirated into the bottles using a mouth aspirator (see Figure 2.5 for bottle bioassay setup). We aimed to have 15 male and 15 female mosquitoes per bottle. The mosquitoes were left in the bottles for 60

min and the number of mosquitoes knocked down was recorded every 10 min. Knockdown behavior was identified as mosquitoes that were on their backs and unable to right themselves. After 60 min, the mosquitoes were transferred to recovery cups, provided cotton balls soaked in a 10% sucrose solution and were left in insectary conditions. After 24 h, the number of dead mosquitoes was counted and recorded. Mosquitoes were counted as dead if they were motionless, if they were on their backs and could not right themselves, or if they were unable to fly.

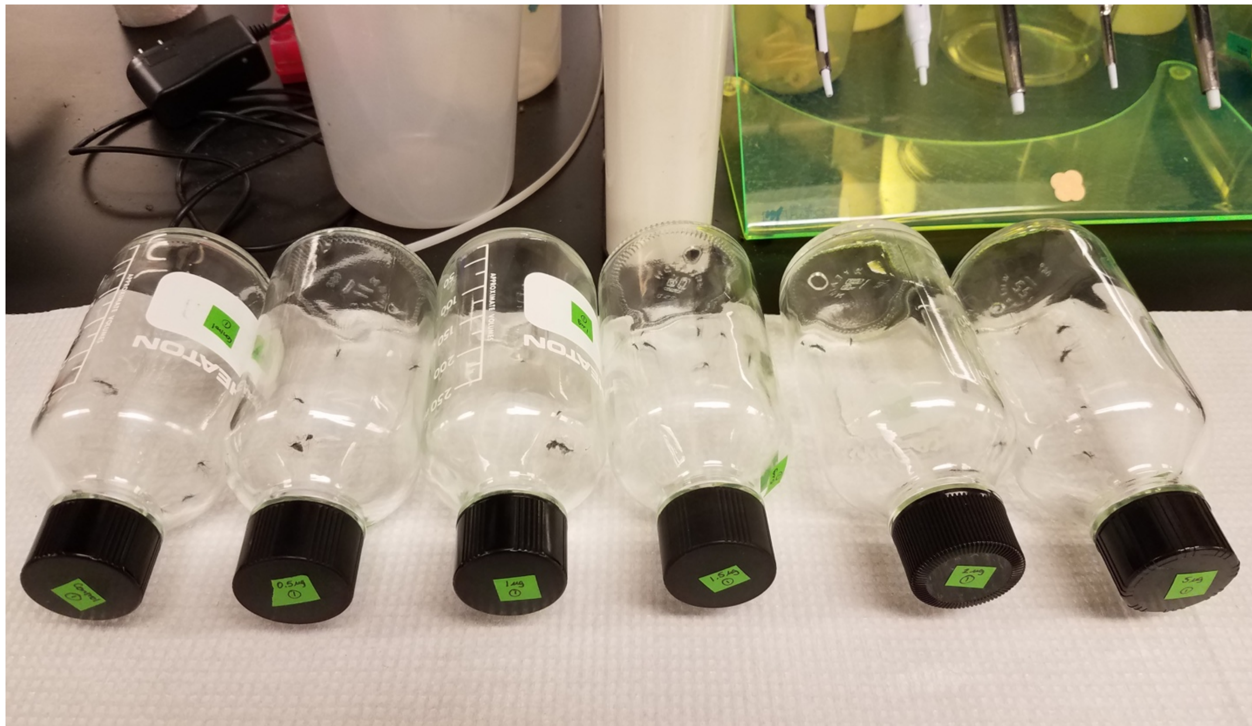

Figure 2.5: Permethrin bottle assay with *Ae. albopictus* mosquitoes. The control bottle is on the left, and the other bottles are ordered (left to right) as follows: 0.5, 1.0, 1.5, 2.0, and 5.0 μg/bottle. For every mosquito strain, each concentration was tested in triplicate. For each replicate, we used different bottles (with the same concentration). Photo by Ashley Janich.

It should be noted that this procedure was slightly different for the initial testing of the 11 colonies from M.X. For testing of these colonies, we counted the number of dead mosquitoes 4 h post-assay so that dead individuals could be frozen and sequenced to compare differences in

mutations in the voltage gated sodium channel (VGSC) gene between the dead, recovered, and alive mosquitoes. However, due to time constraints, it was not possible to complete these experiments. Therefore, for the subsequent testing of the south Texas mosquito populations, we counted all of the dead mosquitoes after 24 h, and disregarded the recovered mosquitoes in order to complete the assays more efficiently and quickly.

Statistical Analysis of Bottle Bioassays

The lethal concentration needed to kill 50% of the mosquitoes ($LC_{50}$) and the 95% highest density intervals (95% HDI) were calculated for every strain tested by using Bayesian analysis on SAS software. Resistance ratios (RRs) and intervals were calculated by dividing the $LC_{50}$ and 95% HDI intervals of the test strain with the $LC_{50}$ and 95% HDI intervals of the control strain (ATM-NJ95). A RR $\leq 1$ would imply that the test strain is equal or more susceptible to permethrin than the control strain. If the RR and its interval were $> 1$, then they were considered to have low levels of resistance relative to the control strain. The ATMNJ95 control strain was assayed multiple times throughout our study. Since we could not test all the mosquito strains at one given time, we had to re-assay ATMNJ95 every time we assayed a set of test strains together. Refer to Appendix Table A.2.2 to see how the strains were grouped together and the $LC_{50}$ values of each ATMNJ95 assay that was used to calculate the RRs of each group.

Artificial Permethrin Selection

We created a laboratory permethrin-selected strain, which from this point on will be referred to as the La Macha strain. The 11 separate colonies from M.X. were tested via bottle assays at the $F_3$ generation, and then the survivors from the assays were kept and allowed to

interbreed with each other. In other words, we took the surviving mosquitoes from all 11 sites and pooled them together into La Macha. Additionally, we also used this same pool of mosquitoes to create a non-selected strain known as La Delicada. The mosquitoes of the La Delicada group were exposed to permethrin at the $F_3$ generation, prior to pooling the collection sites. However, the major difference between the two groups was that La Delicada had no further exposure to permethrin from that point on, while La Macha was repeatedly selected with permethrin every other generation. Specifically, La Macha adults were tested and selected with permethrin at the $F_5$, $F_7$, $F_9$ and $F_{11}$ generations.

Selection was carried out using the same bottle assay procedures and statistical analyses as described in the previous sections, which means that the mosquitoes were pressured with a range of concentrations, rather than a single concentration. There were two main reasons behind this methodology. One reason was to assure that we were not applying too great of a selection pressure on the mosquitoes, in order to prevent the population from crashing. The other reason was to reduce the amount of time and work required for the project, because every time we performed a bottle assay for La Macha, not only were we obtaining necessary data, but we were also simultaneously selecting for resistance.

**Results**

Baseline Permethrin Resistance of *Aedes albopictus* from Mexico and Texas

Overall, the *Ae. albopictus* in this study had low levels of resistance relative to the control strain. Out of the field sites from M.X., the mosquitoes from site G (Col. 5 Febrero) had the highest RR, 2.00 (1.76 – 2.40), while mosquitoes from Puerto Madero had the lowest RR, 1.18 (0.93 – 1.44) (Figure 2.6 and Table A.2.2). The RR of site G was significantly higher than the

RRs of F (Los Llanes), H (San Agustin), Puerto Madero, Huixtla and Esquintla. There were no distinct resistance patterns observed when comparing sites from within the city of Tapachula to rural/suburban sites outside of the city, nor did we observe a trend of decreasing RR with increasing distance from the city (Figure 2.6). The *Ae. albopictus* from Texas also had low levels of resistance relative to the control strain. Mosquitoes from WCC had the highest RR, 2.40 (2.09 – 3.11), while WM10 had the lowest RR, 1.37 (1.28 – 1.59). The RR of WM10 was significantly lower than the RRs of WCC and ELG (See Figure 2.7 and Table A.2.2).

Artificial Permethrin Selection

The La Macha strain was selected with permethrin a total of five times, starting with the $F_3$ generation (prior to pooling the separate colonies) and at the $F_5$, $F_7$, $F_9$ and $F_{11}$ generations. The $F_5$ generation had a RR of 1.75 (1.68 – 1.86), the $F_7$ had a RR of 2.04 (1.71 – 2.75), the $F_9$ generation had a RR of 2.26 (1.99 – 3.15), and the $F_{11}$ generation had a RR of 1.28 (1.23 – 1.44) (Figure 2.6 and Table A.2.2). The $F_5$, $F_7$, and $F_9$ generations all demonstrated an increasing trend in RRs over time, with the $F_9$ generation being significantly higher than the $F_5$ generation. However, the RR significantly dropped at the $F_{11}$ generation. The 95% HDI of the La Macha $F_{11}$ generation did not overlap with any of the other previously selected generations.

The La Delicada strain was last exposed to permethrin at the $F_3$ generation (prior to pooling the separate colonies) but was not exposed to insecticides from that point on. We first tested La Delicada at the $F_9$ generation to presumably allow enough time without exposure to insecticides to potentially see a decrease in the RR from what was observed in the separate field populations. The $F_9$ generation of La Delicada had a RR of 1.17 (1.13 – 1.27) and the $F_{11}$ generation had a RR of 1.53 (1.49 – 1.81) (Figure 2.6 and Table A.2.2). The 95% HDI did not

overlap for the two La Delicada generations, which indicated a significant difference between the two groups.

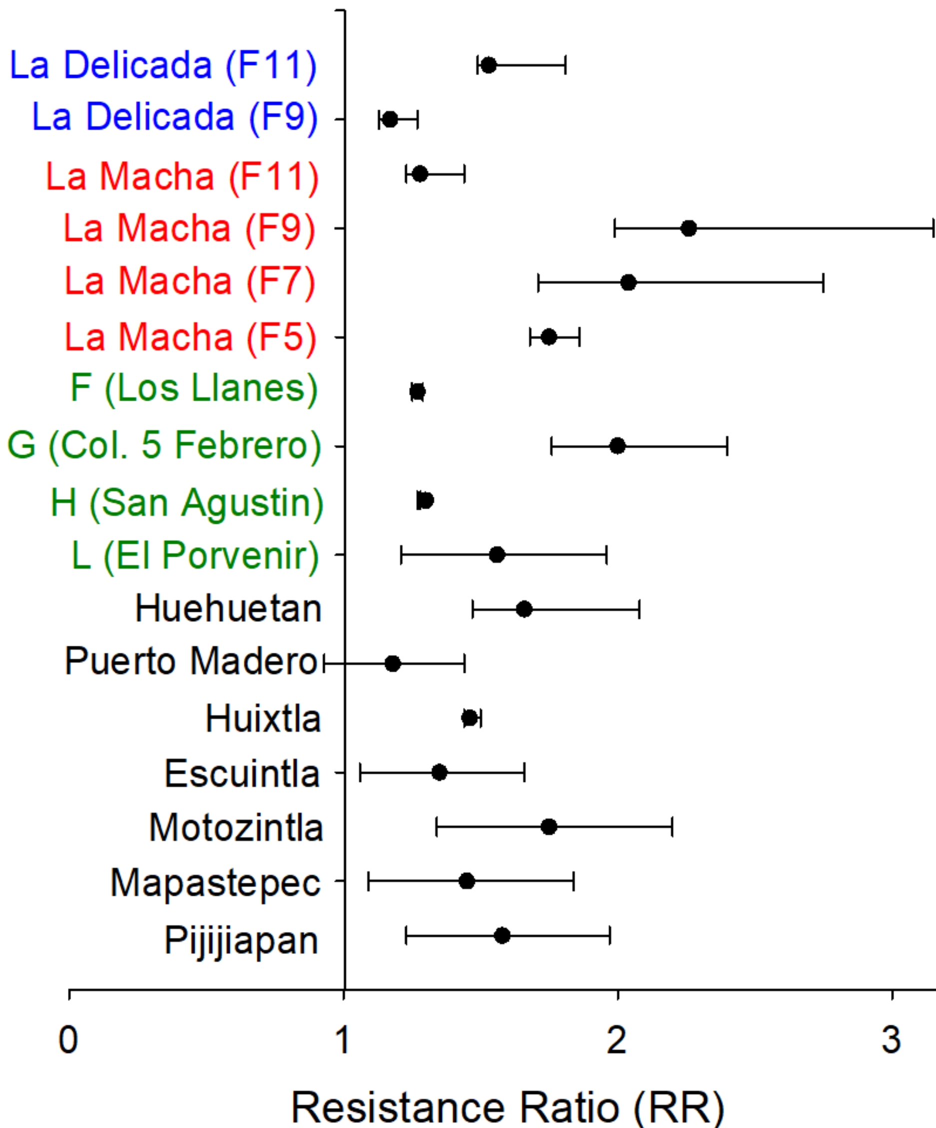


Figure 2.6: Plot of Resistance Ratios (RRs) and intervals among the separate field sites from M.X. The colonies from outside of Tapachula are in black and listed in descending order from closest to the city to furthest from the city. The colonies from Tapachula are in green and are listed alphabetically. The selected (La Macha) and non-selected (La Delicada) Mexican populations are in red and blue, respectively, and are listed in descending order of generation number. RRs and intervals were calculated by dividing the $LC_{50}$ and 95% HDI of the test strains with the $LC_{50}$ and 95% HDI of the control strain (ATM-NJ95). RRs $\leq 1$ would imply the test strain is equally susceptible or more susceptible to permethrin than the control strain. Non-overlapping intervals suggest significant difference between strains.

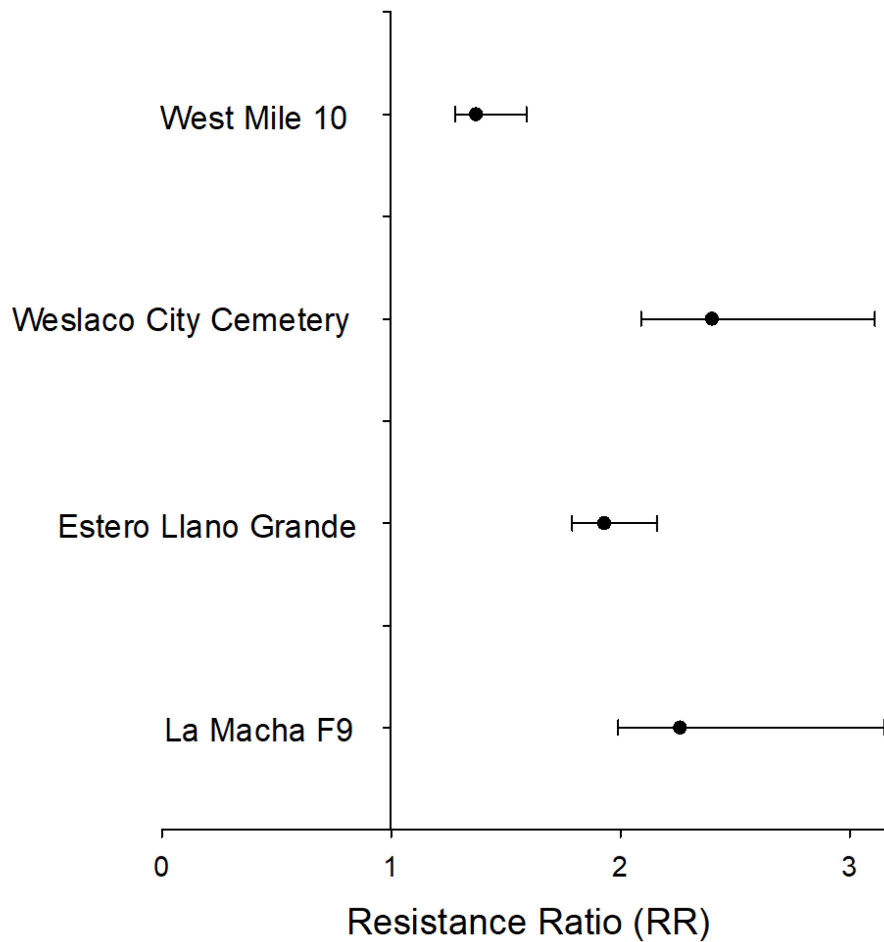


Figure 2.7: Plot of Resistance Ratios (RRs) and intervals among the separate field sites from Texas, which are listed (top to bottom) from northernmost to southernmost locations within the city of Weslaco, and of the selected La Macha $F_9$ (for comparison purposes, since it had the highest RR among the M.X. populations). RRs and intervals were calculated by dividing the $LC_{50}$ and 95% HDI of the test strain with the $LC_{50}$ and 95% HDI of the control strain (ATM-NJ95). RRs ≤ 1 would imply the test strain is equally susceptible or more susceptible to permethrin than the control strain. Non-overlapping intervals suggest significant difference between strains.

**Discussion**

Low Levels of Resistance in Field *Aedes albopictus* Mosquitoes

The *Ae. albopictus* field strains of this study had low RRs, except possibly the Puerto Madero strain, which had a RR of 1.18 (0.93 – 1.44), indicating similar permethrin-susceptibility

with the control strain ATM-NJ95. This contrasts with the higher levels of resistance our lab has observed in the *Ae. aegypti* we collected from the same localities of Texas and Chiapas (unpublished data). However, the results of this study seem fairly consistent with others that have looked at the resistance status of field *Ae. albopictus* mosquitoes, in which they found that the populations either had low-levels of possible pyrethroid-resistance or were susceptible to pyrethroids (Liu et al. 2004, Vontas et al. 2012, Wan-Norafikah et al. 2013, Marcombe et al. 2014, Kushwah, Mallick, et al. 2015, Bengoa et al. 2017, Bharati and Saha 2017, Chatterjee et al. 2018).

Some studies comparing pyrethroid resistance between the two *Aedes* species have also found that *Ae. aegypti* has higher resistance compared to *Ae. albopictus* from the same regions. For example, one study found all 21 of their *Ae. aegypti* strains from Florida were resistant to permethrin with the RRs ranging from 6 to 61, while the 5 *Ae. albopictus* strains from Florida had very low levels of resistance, with RRs ≤ 1.6 (Estep et al. 2018). Even in instances when the two species were collected from the same containers, the *Ae. aegypti* from Florida were much more resistant (Estep et al. 2018). Ponlawat, Scott and Harrington (2005) investigated the susceptibility status of both *Ae. aegypti* and *Ae. albopictus* collected from Thailand in 2003 and 2004 using larval assays. They found that in all of their study sites, *Ae. aegypti* was resistant to permethrin, while *Ae. albopictus* collected from two out of the four sites had low levels of resistance to permethrin, and *Ae. albopictus* from the other two locations were resistant to permethrin (Ponlawat et al. 2005). A few years later, another study in Thailand also found that *Ae. albopictus* were more susceptible to permethrin compared to *Ae. aegypti* collected from the same sites using WHO tube assays with insecticide impregnated papers (Chuaycharoensuk et al. 2011). Based on mortalities, all of the *Ae. aegypti* populations were resistant (mortalities ranged

from 54 – 78%), four *Ae. albopictus* populations were possibly resistant (mortalities ranged from 84 – 96%) and only one *Ae. albopictus* population was resistant (78% mortality) (Chuaycharoensuk et al. 2011). Ishak et al. (2015) observed susceptibility to type I (e.g. permethrin) and type II (e.g. deltamethrin) pyrethroids in populations of *Ae. albopictus* from Malaysia, other than one population from Kuala Lumpur, which was deemed as moderately resistant to permethrin and deltamethrin, but only had mortalities of 87 and 89% respectively. These results contrasted with the *Ae. aegypti* populations they tested from the same region, which all showed resistance to permethrin and deltamethrin, especially those from Kuala Lumpur, in which the mortalities ranged from 0 – 9% (Ishak et al. 2015).

One study found that a small proportion of their mosquitoes, a mixed population of *Ae. aegypti* and *Ae. albopictus* from Port-au-Prince, Haiti, were able to survive the diagnostic dose of permethrin (15 μg) when tested in the field (McAllister et al. 2012). However, the survivors were primarily *Ae. aegypti*, and interestingly, they did not observe phenotypic resistance in this population when the assays were performed in the lab. According to Yu (2015), intrinsic insecticidal toxicity can be affected by the following factors: age, rearing temperature, diet, sex, population density and light exposure. Therefore, McAllister, Godsey and Scott (2012) suggested the difference in resistance levels they observed between the field and lab assayed mosquitoes were likely due to dissimilar environmental temperatures and larval diet, since these were the only two factors that were different between the lab and field settings they tested in. We might also speculate that the resistance levels of the *Ae. albopictus* we tested in this study, and possibly *Ae. albopictus* tested in previous studies, were lower than what would have been observed in the field due to these environmental variables.

It is also possible that *Ae. albopictus* mosquitoes are less resistant in areas they have recently colonized and are more resistant in their native regions. For example, several studies in the U.S. have demonstrated little or no resistance in the populations of *Ae. albopictus* that have been evaluated. Similar to our results, Liu et al. (2004) observed 2- and 4- fold higher resistance to permethrin in two *Ae. albopictus* populations collected from Florida and Alabama (respectively), while the other two collections had similar susceptibility to the control strain, according to the results of their larval bioassays. Additionally, Marcombe et al. (2014) tested several *Ae. albopictus* populations collected from New Jersey, Florida, and Pennsylvania found them all to be highly susceptible to the pyrethroid adulticides they evaluated (deltamethrin, phenothrin and pallethrin). One other study in the U.S. found that only one population of *Ae. albopictus* (collected from North Carolina) was possibly resistant to permethrin, while the other seven populations (from North Carolina, Florida, California and Texas) were all susceptible (Richards et al. 2017). In Spain, another country where *Ae. albopictus* was recently detected (2004), one study found potential resistance to cypermethrin in two out of four *Ae. albopictus* populations, and only one population that was resistant to permethrin and deltamethrin (mortalities ranged from 85 to 95%) (Bengoa et al. 2017).

Higher resistance levels in *Ae. albopictus* populations seem to be more frequently reported in endemic Asian countries, compared to countries where the species recently invaded. Karunaratne et al. (2013) found that *Ae. albopictus* and *Ae. aegypti* from five collection sites in Sri Lanka were all resistant to permethrin (via WHO tube assays). Interestingly, the *Ae. albopictus* from these sites had comparatively lower mortalities (25-54%) than the *Ae. aegypti* (38-60%) (Karunaratne et al. 2013). Another study also found resistance in *Ae. albopictus* mosquitoes and *Ae. aegypti* collected from the same sites in Pakistan. The results of their WHO

tube assays suggested both species were resistant to permethrin, with mortalities ranging from 62 to 74% (Arslan et al. 2016). Their results also suggested *Ae. albopictus* had possible resistance to deltamethrin and lambda-cyhalothrin with mortalities ranging from 90 to 96% (Arslan et al. 2016). To clarify, the interpretation of these mortality ranges were based off of the WHO recommended criteria: mortality ≥ 98% indicates susceptibility, mortality ranging from 90 – 97% suggests probable/possible resistance (further investigation is needed), and mortality < 90% confirms resistance (World Health Organization 2013). In China, Chen et al. (2016) found two urban-sourced populations of *Ae. albopictus* from Hainan Island to have high levels of resistance to permethrin, beta-cypermethrin and deltamethrin (RRs ranged from 8.83 to 436.36). They noted that the three rural populations they tested were largely susceptible to all three pyrethroids by comparison, which they suspected was due to infrequent spraying of insecticides in rural areas compared to urban areas (Chen et al. 2016). Although not nearly as high, Xu et al. (2016) reported possible resistance (96.1% mortality) and resistance (90.1% mortality) to deltamethrin in two populations of *Ae. albopictus* from southern China, and that the resistance observed in these two populations were positively associated with a knock-down-resistant (*kdr*) mutation they found at the 1534 site of the VGSC. See Chapter 3 for further discussion about resistance and the associated mechanisms.

From this study, we conclude that the pyrethroid resistance status in *Ae. albopictus* collected from Southern Texas and Southern Chiapas is low. As of January, 2020, the current diagnostic dose of permethrin recommended by the CDC for *Ae. aegypti* and *Ae. albopictus* mosquitoes is 43 μg/bottle with a diagnostic time of 10 min, which is expected to have a 100% mortality rate (https://www.cdc.gov/zika/vector/insecticide-resistance.html). While this diagnostic dose is specific to bottle assay analysis of resistance in a laboratory setting, and the

actual concentration of permethrin used in the field would be lower depending on the method and delivery of the insecticide, it still allows us to determine if the insecticide is beginning to lose its effectiveness against field populations of mosquitoes. Since the diagnostic dose currently recommended by the CDC is significantly higher than the concentrations we used in this study (recall our highest concentration was 5 μg/bottle), we conclude that permethrin is currently still a viable option for controlling field populations of *Ae. albopictus* in the regions we collected from.

Permethrin Selection

Although we anticipated our field strains to be resistant to permethrin at the start of this project, we could not guarantee this. Therefore, we wanted to test if we could artificially select *Ae. albopictus* to be resistant to permethrin in the laboratory. The La Macha strain initially appeared to increase in resistance, with the RR significantly being higher in the $F_9$ generation compared to $F_5$, and with the $F_9$ RR, 2.26 (1.99 – 3.15), being the highest observed ratio among the tested Mexican populations. However, at the $F_{11}$ generation of La Macha, there was an unexpected and significant drop in the RR (1.28 (1.23 – 1.44)). We also observed the opposite, but equally unexpected situation for La Delicada, where the RR significantly increased from the $F_9$ to the $F_{11}$ generation. The RR of La Delicada $F_{11}$, 1.53 (1.49 – 1.81), was even higher than the RR of La Macha $F_{11}$. We expected that the RR would increase in the La Macha strain with continued insecticide pressure and the RR would decrease or stay roughly the same in the La Delicada strain without further exposure to insecticides. However, our actual results were incongruent with our expectations. A simple explanation for these results could be that these differences are just due to random variation.

However, other studies have also reported failed or contradictory results when artificially selecting for resistance in *Ae. albopictus* mosquitoes. One study briefly mentioned that their attempts to select *Ae. albopictus* collected from Florida with permethrin were unsuccessful (Estep et al. 2018), but they did not report the details of their selection methods. Additionally, there was another group that also tried to select for insecticide resistance in *Ae. albopictus* by pressuring fourth instar larvae with malathion, but they could not increase phenotypic resistance (Selvi et al. 2010). Similar to our results, they also observed a decline in resistance with the selected strain. They suggest that a possible reason for this observation could be due to the heterozygous individuals diluting the gene pool, allowing the susceptible phenotype to dominate the overall population (Selvi et al. 2010). Perhaps this could be a factor contributing to the results we observed with our selected *Ae. albopictus*. Another explanation could be that we simply did not select over enough generations to observe an increase in resistance. For our study, we pressured the La Macha strain a total of five times, starting with the $F_3$ generation, and selected every other generation up to $F_{11}$. While the previously mentioned study (Selvi et al. 2010) only pressured their mosquitoes every five generations and stopped selecting at the $F_{10}$ generation. Perhaps if we were to continue to select the mosquitoes in the lab over enough generations and allow the homozygous resistant individuals (if any are present) to accumulate in the population, the resistance would increase. Future studies would need to confirm this possibility. An additional possibility for the increased mortality in our selected strain could be that the selection pressure inadvertently caused inbreeding within our population. Inbreeding can result in populations with higher proportions of individuals homozygous for deleterious or lethal recessive alleles (Charlesworth and Willis 2009). If so, this strain would have been generally weaker and more susceptible to even low doses of insecticide.

Another group looked at the differences in permethrin-resistance of field *Ae. albopictus* from Malaysia compared to a permethrin-selected strain. Unlike our study, they selected their *Ae. albopictus* with the $LC_{50}$ concentration at every generation and reported the results of the $F_5$ generation (Wan-Norafikah et al. 2013). From their larval assays they found that the permethrin-selected and field *Ae. albopictus* mosquitoes were approximately two times more resistant than the control strain, with RRs ranging from 1.90 – 2.20 (Wan-Norafikah et al. 2013). Similar to our results, they observed low RRs, even with their permethrin-selected strain. But since the RR of the permethrin-selected strain was low and similar to our observed ratios, and since they only carried out five selection events, it is unclear as to whether or not they successfully selected the strain with permethrin. Hamzah and Alias (2016) also compared a permethrin-treated strain to a field *Ae. albopictus* strain from Kuala Lumpur, Malaysia and found that both had very similar RRs ($3.61\pm0.04$ and $3.53\pm0.04$ respectively), however due to ambiguity in the methodology used in deriving the permethrin-treated strain, it is difficult to interpret these results. Overall from multiple studies it appears to be difficult to artificially select resistant *Ae. albopictus* in the lab.

We must also consider how pyrethroid resistance in populations of *Ae. albopictus* has changed over time. For example, a previous study reported *Ae. albopictus* collected from Italy and Greece during the 2000s were susceptible to the diagnostic dose (0.05%) of deltamethrin-impregnated papers (Vontas et al. 2012). However, a later study found that *Ae. albopictus* collected from Italy and Greece in 2016 had varied levels of resistance to permethrin and α-cypermethrin, which was the first report of pyrethroid resistance in Italian *Ae. albopictus*, but they were still susceptible to deltamethrin (Pichler et al. 2018). Additionally, Kamgang et al. (2011) evaluated resistance in *Ae. aegypti* and *Ae. albopictus* populations collected from Central Africa in 2007 and only found one *Ae. albopictus* population to have suspected resistance to

deltamethrin. However, in 2015 and 2016, Kamgang et al. (2017) studied the resistance of *Ae. aegypti* and *Ae. albopictus* collected from the same region in Africa. They found that both species were resistant to deltamethrin, and both species were susceptible to permethrin, except for two of the *Ae. albopictus* populations collected during the rainy season (Kamgang et al. 2017). It is possible that the observed differences of *Ae. albopictus* resistance in the aforementioned studies were a result of selection pressure in the field over time.

One other possible reason for why we observed lower levels of resistance in *Ae. albopictus* could be because of species-specific traits. For example, Liu et al. (2004) observed that *Culex quinquefasciatus* had higher resistance to more insecticides compared to *Ae. albopictus* from the same sites. They suggested this interspecies difference could be contributed to the fact that *Cx. quinquefasciatus* is native to the regions and has had longer exposure to insecticides than *Ae. albopictus*, or it could also be due to ecological and behavioral differences between the two species, resulting in different levels of insecticide exposure (Liu et al. 2004). This idea is further supported by a recent study that was conducted in an urban park of São Paulo, Brazil, in which they found that the distributional range of *Ae. aegypti* was more frequently associated with the periphery of the park, which was surrounded by an urban setting; whereas the *Ae. albopictus* were more frequently distributed in the interior portions of the park where there was more vegetation, reaffirming previous reports of habitat preference of the two species (Heinisch et al. 2019) and a likelihood that the two species have dissimilar exposure to insecticides. We might also consider that neighborhood configurations could potentially affect how much mosquitoes are exposed to insecticides. Sames IV et al. (1996) suggested that due to the house configurations within their study sites, mosquitoes in the backyards were protected from the insecticides being sprayed from vehicles on the street, which possibly could help

maintain a susceptible population of mosquitoes. While we did not make note of the neighborhood and house configurations within our study sites, perhaps this could be a factor contributing to the susceptible levels observed in our *Ae. albopictus*. It would be beneficial to conduct similar studies evaluating the variation of spatial distribution between the two *Aedes* species in southern M.X. and Texas to see if the *Ae. albopictus* are primarily distributed in areas where there is reduced or no exposure to insecticides.

Additionally, since *Ae. aegypti* are more highly associated with human dwelling spaces, it is possible that *Ae. aegypti* mosquitoes receive more exposure, and therefore more selection pressure, to household insecticide sprays compared to *Ae. albopictus*. Evidence for such selection was demonstrated by Gray et al. (2018) when they compared the efficacy of household pyrethroid-based sprays between a susceptible population and 3 pyrethroid-resistant field strains of *Ae. aegypti*, and found that mortality was significantly lower when the insecticides were applied to the resistant strains. They also observed a higher frequency of individuals homozygous for the I1016 mutation among those that survived the insecticide exposure (Gray et al. 2018). Selection for resistance could be occurring within households more than we realize. One study found that a high majority (87%) of surveyed households in the dengue-endemic area of Yucatan State in Southern M.X. took personal action to kill mosquitoes and other pests, and of those households, the majority of them (73.6%) chose to use aerosol insecticide sprays (Loroño-Pino et al. 2014). Perhaps similar action is being taken by households in other dengue-endemic areas. It would be useful to do more broad-based surveys to see what and how household insecticide products are being used to target mosquitoes in other regions of M.X. and the U.S. to determine if this is a factor contributing to resistance in certain mosquito species.

It is worth noting that *Ae. aegypti* and *Ae. albopictus* collected from the southern tip of Texas in 1995 were found to be susceptible to organophosphates and type I pyrethroids (Sames IV et al. 1996). The methods of testing for resistance have changed since this study, so our results can only be loosely compared to this previous analysis of resistance. However, future studies should re-evaluate and monitor the resistance status of the *Ae. albopictus* and *Ae. aegypti* collected from our sites in Texas and Chiapas to give a better longitudinal perspective on the insecticide resistance status in these locations.

# CHAPTER 3 – INVESTIGATION OF POSSIBLE MECHANISMS CONFERRING RESISTANCE IN LESS SUSCEPTIBLE *AEDES ALBOPICTUS* POPULATIONS

## Introduction

The two main mechanisms conferring resistance to insecticides are target site mutations and detoxifying enzymes. Multiple mutations in the voltage-gated sodium channel (VGSC) gene are suspected of conferring pyrethroid resistance in *Ae. aegypti* mosquitoes. Non-synonymous mutations in these genes change the amino acid encoded, thereby altering the conformation of the protein to prevent binding of the insecticide to the sodium channel of the nerve cell (Saavedra-Rodriguez et al. 2007). These mutations include G923V, L982W, I1011M/V, V1016G/I (Brengues et al. 2003, Saavedra-Rodriguez et al. 2007), D1763Y (Chang et al. 2009), S989P (Srisawat et al. 2010), F1534C (Yanola et al. 2011), T1502I (Kushwah, Dykes, et al. 2015) and V410L (Haddi et al. 2017, Saavedra-Rodriguez et al. 2018). Mutation sites are numbered according to the codon position in the house fly (*Musca domestica*) sodium channel gene, flanked by the original and new amino acid letter designations (we chose to use this numbering system to remain consistent with previous studies). These mutations have been globally recognized as "knock-down resistant" or "*kdr*" mutations due to their ability to prevent mosquitoes and other insects from exhibiting knock down behavior (rapid twitching, inability to fly) when they are exposed to pyrethroids (Saavedra-Rodriguez et al. 2007). However, only several of these mutations have been confirmed to functionally decrease the VGSC sensitivity to pyrethroids in *Ae. aegypti*, namely S989P (located in DIIL5-6), I1011M, V1016G (DIIS6), F1534C (DIIIS6), and V410L (DIS6) (Du et al. 2013, Saavedra-Rodriguez et al. 2018). Recall from Chapter 1 that the VGSC is comprised of four domains (DI – DIV), each consisting of six

segments (S1 – S6) connected by linker helices (L), which are also used to describe the physical location of the mutations within the VGSC (See Figure 3.1).

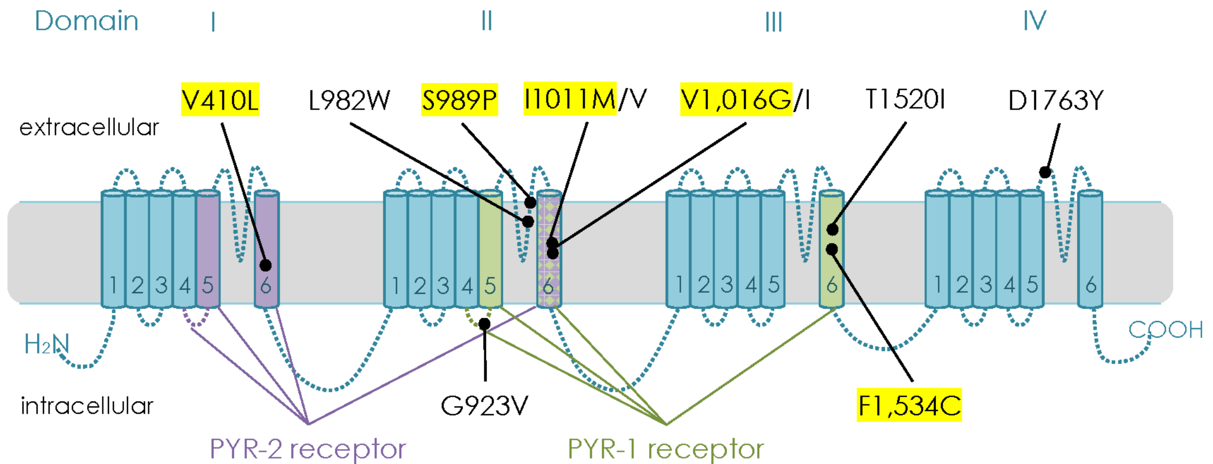


Figure 3.1: Diagram of locations of *kdr* mutations in the VGSC of *Ae. aegypti*. Mutation positions are numbered according to the housefly sodium channel gene. Domains I-IV are shown and the membrane spanning segments within each domain are numbered. Linker helices connecting the segments are shown as dotted lines. The mutations highlighted in yellow are the ones that have been confirmed to functionally reduce sensitivity of the VGSC to pyrethroids. The suspected binding sites of pyrethroids, pyrethroid receptors 1 and 2, are shown in green and purple, respectively. Figure is modified from Saavedra-Rodriguez et al. (2018).

Within the last decade, several VGSC mutations associated with resistance to pyrethroids have been found in *Ae. albopictus*. The first reported mutation, F1534C, was found in high frequency in *Ae. albopictus* collected from Singapore in 2009 (Kasai et al. 2011). Since then, this mutation has also been reported in *Ae. albopictus* from Greece (Xu et al. 2016), China (Chen et al. 2016, Gao et al. 2018), Brazil (Aguirre-Obando et al. 2017) and Vietnam (Kasai et al. 2019). Other suspected *kdr* mutations have also been found in the codon for amino acid 1534 of the VGSC in *Ae. albopictus*. For example, F1534L and F1534S mutations have primarily been observed in *Ae. albopictus* from China (Chen et al. 2016, Xu et al. 2016, Gao et al. 2018, Li et al. 2018, Zhou et al. 2019), but F1534L has also been reported in one *Ae. albopictus* mosquito from

Florida (Marcombe et al. 2014) and recently F1534S was also reported in *Ae. albopictus* collected from Vietnam (Kasai et al. 2019). One other mutation in DIIIS6, I1532T, has also been found in *Ae. albopictus* from China (Gao et al. 2018, Zhou et al. 2019) and Italy (Xu et al. 2016). Outside of DIII, very few mutations have been reported in the other VGSC domains in *Ae. albopictus*. Multiple studies have searched for possible resistance-conferring mutations in DII and DIV of *Ae. albopictus* but did not find any (Kasai et al. 2011, Marcombe et al. 2014, Kushwah, Mallick, et al. 2015, Xu et al. 2016, Aguirre-Obando et al. 2017, Li et al. 2018). However, McAllister, Godsey and Scott (2012) did find I1011M and I1011V mutations (DIIS6) in high frequency in populations of both *Ae. aegypti* and *Ae. albopictus* from Port-au-Prince Haiti. Aside from that, the only other mutation that has been reported in DIIS6 is V1016G in *Ae. albopictus* from Italy, Vietnam (Kasai et al. 2019) and China (Zhou et al. 2019). See Figure 3.2.

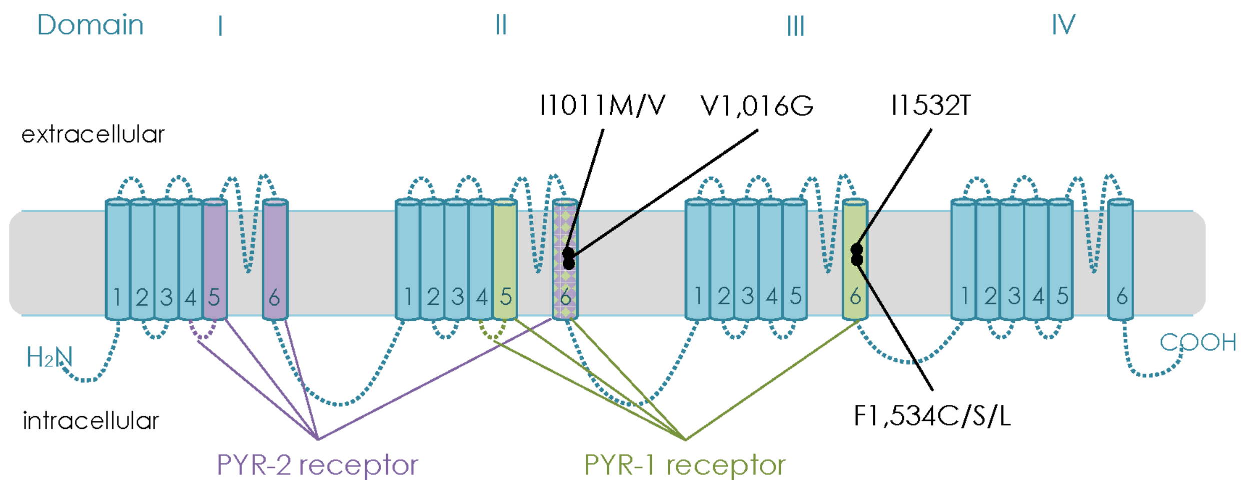


Figure 3.2: Diagram of locations of *kdr* mutations in the VGSC protein that have been found in *Ae. albopictus*. Mutation positions are numbered according to the housefly sodium channel gene. Domains I-IV are shown and the membrane spanning segments within each domain are numbered. Linker helices connecting the segments are shown as dotted lines. The suspected binding sites of pyrethroids, pyrethroid receptors 1 and 2, are shown in green and purple, respectively. Figure is modified from Saavedra-Rodriguez et al. (2018).

While these mutations have yet to be confirmed in their functional ability to reduce the sensitivity of the VGSC to pyrethroids (confer resistance) in *Ae. albopictus*, multiple studies demonstrated that these mutations were associated with pyrethroid resistance in the species. Several groups have found an association of the F1534S (*kdr*) mutation with pyrethroid resistant *Ae. albopictus* from China (Xu et al. 2016, Gao et al. 2018, Li et al. 2018). Li et al. (2018) also found an association of the F1534L mutation to pyrethroid resistant *Ae. albopictus*. However, this contrasts with the results from Gao et al. (2018) and Xu et al. (2016), which both found no association between pyrethroid resistance and the F1534L mutation. Recently, the V1016G mutation was detected in high frequency in *Ae. albopictus* from Italy and Vietnam and was also associated with pyrethroid-resistant populations (Kasai et al. 2019).

Aside from *kdr* mutations in the VGSC discussed in the previous paragraphs, several major groups of enzymes have also been implicated in metabolic resistance to pyrethroids: cytochrome P450 monooxygenases (P450s; a.k.a. mixed function oxidases), glutathione *S*-transferases (GSTs) and esterases. Within insects, P450s are involved in detoxifying and/or modifying endogenous and xenobiotic (foreign chemicals) compounds and are associated with resistance to many insecticide classes, including pyrethroids, organophosphates, organochlorines and carbamates (Hemingway et al. 2004, Yu 2015). Resistance can occur from duplications of genes that encode P450s or by transcriptional upregulation of P450s, which increase the amount of enzyme that is produced, and thereby increase enzymatic activity, which results in the production of less toxic metabolites (Liu 2015, Yu 2015). GSTs are able to detoxify, metabolize and excrete endogenous and xenobiotic compounds and have been associated with resistance to all major insecticidal classes (Enayati et al. 2005). Resistance mediated by GSTs also occurs when the enzymes are produced in higher quantities, either from gene duplication or

transcriptional upregulation (Hemingway et al. 2004, Enayati et al. 2005). Esterases typically belong to the carboxylesterase gene family and they play major roles in developmental and behavioral processes of insects by hydrolyzing ester bonds of chemicals, such as pheromones (Montella et al. 2012). They also assist with detoxification of xenobiotic compounds and have been associated with resistance to pyrethroids, organophosphates and carbamates (Hemingway et al. 2004, Montella et al. 2012). Insecticide resistance results from increased esterase activity, which primarily occurs by esterase gene duplication, but can also occur from transcriptional upregulation (Hemingway et al. 2004).

Based on the association between mutations at the 1534 site and pyrethroid resistance in both *Ae. aegypti* and *Ae. albopictus* mosquitoes, the first objective of this study was to investigate whether or not these mutations were associated with insecticide resistant phenotypes in *Ae. albopictus* populations in Southern Mexico and Texas. Specifically, we chose to search for the F1534C mutation because it reduces the sensitivity of the VGSC to pyrethroids, this mutation has reached fixation in several populations of *Ae. aegypti* in Southern Mexico (Vera-Maloof et al. 2015), and it was the first VGSC mutation that was detected in *Ae. albopictus* (Kasai et al. 2011). Secondly, we aimed to investigate if there were increased activities of resistance-conferring enzymes in our less susceptible *Ae. albopictus* strains. In order to do this, we used biochemical assays to measure absorbance values from enzyme reactions of our tested mosquitoes and converted the values into quantifiable measurements of enzyme activity. It is important for us to more clearly understand if and how these mechanisms confer resistance in *Ae. albopictus* mosquitoes in order to prevent the development or maintenance of insecticide resistance in the field.

**Materials and Methods**

Mosquitoes

Mosquito collections undergoing molecular and biochemical analyses in these studies are described in Chapter 2.

Sequencing and Genotyping *kdr* Mutations

We attempted to obtain a partial sequence of exon 29 (contains amino acid site 1534) from our *Ae. albopictus* strains to find mosquitoes with and without the C1534 mutation in order to optimize our allele specific polymerase chain reaction (A*S*-PCR) protocol for the species. *Aedes albopictus* mosquitoes were frozen at -80°C. DNA was extracted from 25 females and 25 males of each colony by salt extraction method using Pat Roman's grinding buffer (0.1 M NaCl, 0.2 M sucrose, 0.1M Tris Buffer, 0.05 M EDTA, 0.5% SDS, pH 9.2) and 8 M Potassium acetate (Black and DuTeau 1997). DNA pellets were resuspended in 1.5 mL tubes with 180 µL of TE buffer (1 M Tri*S*-HCl, 0.5 M EDTA, pH 8.0) and were stored at -80°C until needed.

Initial screening for mutations at the 1534 site of the VGSC was performed by Sanger Sequencing. Samples were first prepared in low 96-well clear Multiplate® PCR Plates™ (Bio-Rad Laboratories, Hercules, CA, USA) by mixing 1 µL of DNA with 24 µL of master mix, which consisted of 12.5 µL GoTaq Green Master Mix (Promega, Madison, WI, USA), 11.4 µL ddH$_2$O, and 0.05 µL of both the forward and reverse primers, aegSCF7 and aegSCR8 (See Table 3.1), per reaction (primers were diluted in TE at a final concentration of 500 pmol/µL). A drop of mineral oil was added to each PCR well, which were then covered with Optical Flat 8-Cap Strips (Bio-Rad Laboratories) and placed in the MyCycler™ thermal cycler (Bio-Rad Laboratories). Partial fragments of DIIIS6 were first amplified with the following thermal cycling conditions:

95°C for 5 min (first denature), 33 cycles of 95°C for 30 s (denature in cycle), 52°C for 30 s (annealing), 72°C for 1 min (extension), followed by a final extension at 72°C for 5 min. PCR products were checked for quality by running 5 µL of product in a 2.0% agarose gel at 90 V for 30 min. PCR products were then purified using the MiniElute® Qiagen Purification kit. Purified samples were premixed with IIIS6short+ and IIIS6short- primers (Table 3.1) and were sent to Genewiz® for Sanger Sequencing (See Figure 3.3 for the partial annotated sequences of exons 28 and 29 of *Ae. albopictus* showing where each primer was aligned for the sequencing and genotyping procedures described herein). The forward and reverse sequences were aligned using Geneious 7.1.7 software (http://www.geneious.com/). We obtained successful sequences from six samples of ATMNJ95 $F_{17}$ females, three samples of Motozintla $F_1$ females, and five samples of La Macha $F_{11}$ females that survived the last bottle assay, all of which had the susceptible genotype (F/F homozygote, see Figure 3.4). Sequences were deemed "successful" if $> 90\%$ pairwise alignment occurred between the base pairs (bp) of the forward and reverse sequences (which totaled ~203-278 bp alignment) of the sample (i.e. good consensus identity, see Figure 3.4). We decided to initially screen for the mutation at site 1534 in the surviving La Macha $F_{11}$ females with the hypothesis that this would be our most resistant strain of *Ae. albopictus*, and therefore would be the strain that would most likely contain individuals with a mutation conferring resistance.

Table 3.1: List of primer sequences used for Sanger Sequencing and A*S*-PCR protocols to screen for mutations at the 1534 site of the Domain III Segment 6 region of the VGSC for *Ae. albopictus*. For the A*S*-PCR primers, the grey highlighted sequences are the long and short GC tails assigned to the forward primers for the cysteine mutation sequence and the phenylalanine wild type sequence, respectively. The green highlighted nucleotides in the forward primers indicate a synonymous polymorphic site where either a C or T could occur in *Ae. albopictus*. Nucleotides highlighted in blue are intentionally mismatched nucleotides to help improve assay specificity. The allele-specific nucleotides are highlighted in pink (G codes for cysteine, T codes for phenylalanine).

| Primer function | Primer name | Primer sequence | Product size (bp) |
|---|---|---|---|
| Amplification primers | aegSCF7 | 5'-GAGAACTCGCCGATGAACTT-3' | 413-429 |
| | aegSCR8 | 5'-TAGCTTTCAGCGGCTTCTTC-3' | |
| Sequencing primers | IIIS6short+ | 5'-AACGATCGTTTCTCTTGA-3' | 172 |
| | IIIS6short- | 5'-CCGGCTTTCTTCTTCTGC-3' | |
| A*S*-PCR primers | C1534_albo | 5'-GCGGGCAGGGCGGCGGGGGCGGGGCCTCTACTTYGTGTTCTTCATCATGTG-3' | 113 |
| | F1534_albo | 5'-GCGGGCTCTACTTYGTGTTCTTCATCATATT-3' | 93 |
| | 1534rev_albo | 5'-TCTGCTCGTTGAAGTTGTCGAT-3' | |

**Partial exon 28 (*Aedes albopictus* VGSC, vector base =AAFL000723)**
Residue 1,414

E N S P M N F D H V G K A Y L C L F Q V

GAGAACTCGCCGATGAACTTCGACCACGTGGGGAAGGCGTACCTGTGTCTGTTCCAGGTG

*aegSCF7(fwd) primer*

A T F K G W I Q I M N D A I D S R E

GCAACGTTCAAGGGCTGGATCCAGATCATGAACGATGCCATCGACTCGCGGGAGgtaagt

**intron 28-29 (67-83 bp)** deletion

tcgggatcttcgatcatcwcatcagttcagccccraatcaatycgaytaacgatcgtttc

*IIIS6short+ primer*

**Exon 29**

V G K Q P I R E T N I Y M Y

tcttgawccctcsrcagGTGGGCAAGCAGCCRATYCGCGAGACCAACATCTACATGTAC

**F1,534 (F1,474)**

L Y F V F F I I F G S F F T L N L F I

CTCCTATTYGTGTTCTTCATCATCTTCGGGTCGTTCTTCACCCTYAAYCTGTTCATC

5′[L-]CTCCTATTYGTGTTCTTCATCATCTG-3′ Cys1,534

5′[S-]CTCCTATTYGTGTTCTTCATCATCTT-3′ Phe1,534

*Forward AS-PCR primers*

G V I I D N F N E Q K K K A G G S L E M

GGTGTCATCATCGACAACTTCAACGAGCAGAAGAAGAAAGCCGGTGGCTCGCTGGAAATG

3′-TAGCTGTTGAAGTTGCTCGTCT-5′ *IIIS6short- primer*

*Reverse AS-PCR primer*

F M T E D Q K K Y Y N A M K K M G S K K

TTCATGACGGAGGATCAGAAAAAGTACTACAACGCAATGAAAAAGATGGGCTCGAAGAAG

3′-CTTCTTC

P L K A I

CCGCTGAAAGCTA

GGCGACTTTCGAT-5′

*aegSCR8 (rev) primer*

Figure 3.3: Partial annotated sequence of exons 28 and 29 in *Aedes albopictus* and the corresponding amino acids (abbreviated letters above gene sequence) encoded by each codon (intron 28-29 is labeled and indicated by lower case letters). Primers used for Sanger Sequencing and A*S*-PCR genotyping protocols are also shown and labeled. The allele-specific nucleotides of the forward A*S*-PCR primers are highlighted in pink (G codes for cysteine, T codes for phenylalanine). Blue highlighted nucleotides indicate a synonymous polymorphic site. Figure by Dr. Karla Saavedra-Rodriguez.

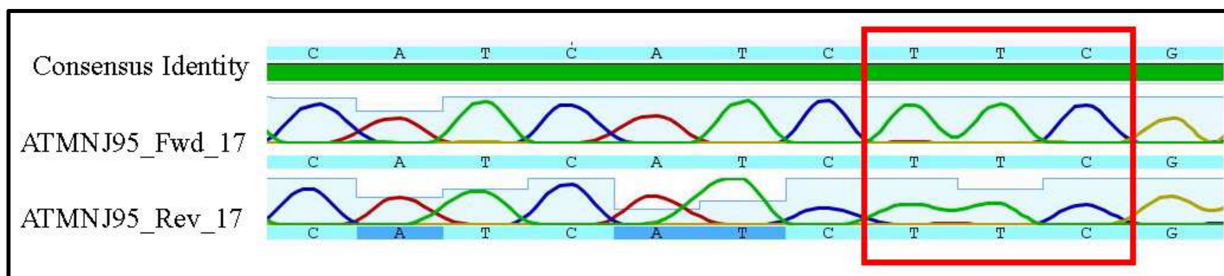


Figure 3.4: Alignment of forward (ATMNJ95_Fwd_17) and reverse-complimented (ATMNJ95_Rev_17) sequences from ATMNJ95 Female #17 ($F_{17}$) using Geneious 7.1.7 software, showing a portion of the DIIIS6 sequence of exon 29. The susceptible wild type codon that codes for phenylalanine is outlined by the red box.

Mosquitoes sampled from the $F_1$ generations of the 11 sites in M.X., the $F_{11}$ La Macha (survivors from bottle assays), $F_{11}$ La Delicada and $F_{17}$ ATMNJ95 were genotyped using the A*S*-PCR protocol, modified from Saavedra-Rodriguez et al. (2007) and Yanola et al. (2011). Allele specificity was increased by intentionally mismatching the third nucleotide from the 3' end of the A*S*-PCR primers (Table 3.1) following the recommendations of Okimoto and Dodgson (1996) and Saavedra-Rodriguez et al. (2007). Long and short GC tails were attached to the 5' end of the A*S*-PCR primers to distinguish the amplified products based on size (Germer and Higuchi 1999, Yanola et al. 2011). The long tail was assigned to the C1534_albo primer that would amplify sequences containing the cysteine mutation and the short tail was assigned to the F1534_albo primer that would amplify sequences containing the phenylalanine wild type codon (Table 3.1). Due to the differing lengths of the primers from the GC tails, different melting curve peaks are observed depending on the sequence that gets amplified. Individuals homozygous for the cysteine mutation would have a melting curve peak at 85°C, while phenylalanine homozygotes would have a melting curve peak at 80°C, and heterozygous individuals would have peaks at both temperatures.

A*S*-PCR samples were prepared in 96-well white Multiplate® PCR Plates™ (Bio-Rad Laboratories) by mixing 1 µL of DNA with 19.6 µL of PCR mix (9.53 µL of $ddH_2O$, 10.0 µL of

iQ SYBR Green Supermix (Bio-Rad Laboratories), 0.066 μL of C1534_albo primer, 0.20 μL of F1534_albo and 0.20 μL of 1534rev_albo primer, per reaction). Primers were suspended in TE and diluted to 50 pmol/μL. Wells were covered with Optical Flat 8-Cap Strips (Bio-Rad Laboratories), centrifuged for 2 min and placed in the CFX Connect™ Real Time System thermal cycler (Bio-Rad Laboratories). The plates were run under the following thermocycler conditions: 95°C for 3 min (first denature), 39 cycles of 95°C for 10 s (denature in cycle), 57°C for 10 s (annealing), 72°C for 30 s (extension), followed by 95°C for 10 sec. Melting curves were obtained by measuring fluorescent reads of the plate as the thermocycler ramped up from 65°C to 95°C in 0.5°C increments every 5 sec. Melting curve peaks were analyzed with Bio-Rad CFX Maestro software.

Since we did not identify any *Ae. albopictus* mosquitoes with the C1534 mutation in our initial sequencing methods, the A*S*-PCR assay was optimized with a synthetic sequence containing the C1534 mutation as a positive control (C1534_seq; ordered from Genewiz®, See Appendix Table A.3.1 for amplicon sequence). The control C1534_seq was mixed with TE (15ng/μL concentration) to ensure we could detect the mutation sequence by observing a melting curve peak at 85°C. We optimized identification of the susceptible genotype with DNA from ATMNJ95 Female #17 ($F_{17}$) to observe a melting curve peak at 80°C to confirm the susceptible genotype could be detected. We chose to use DNA from this individual because we confirmed that it was homozygous susceptible during our initial sequencing of the gene (Figure 3.4). Identification of the heterozygous genotype was optimized by mixing equal parts of susceptible DNA (ATMNJ95 Female #17) with C1534_seq (diluted to 0.0015 ng/μL), to ensure two melting curve peaks were observed. The C1534_seq had to be diluted for the heterozygous mixture in order to observe both peaks (concentrations above 0.0015 ng/μL were too high and only the

cysteine peak was visible). Each of these “control” samples were analyzed on every plate with the test strain mosquitoes to ensure that the protocol was working and that all three genotypes could be identified from the resulting melting curves. See Figure 3.5 for melting curve peaks of control samples. Additionally, all three control samples were run on 3.5% agarose gel to confirm appropriate sequence lengths were obtained (Figure 3.6).

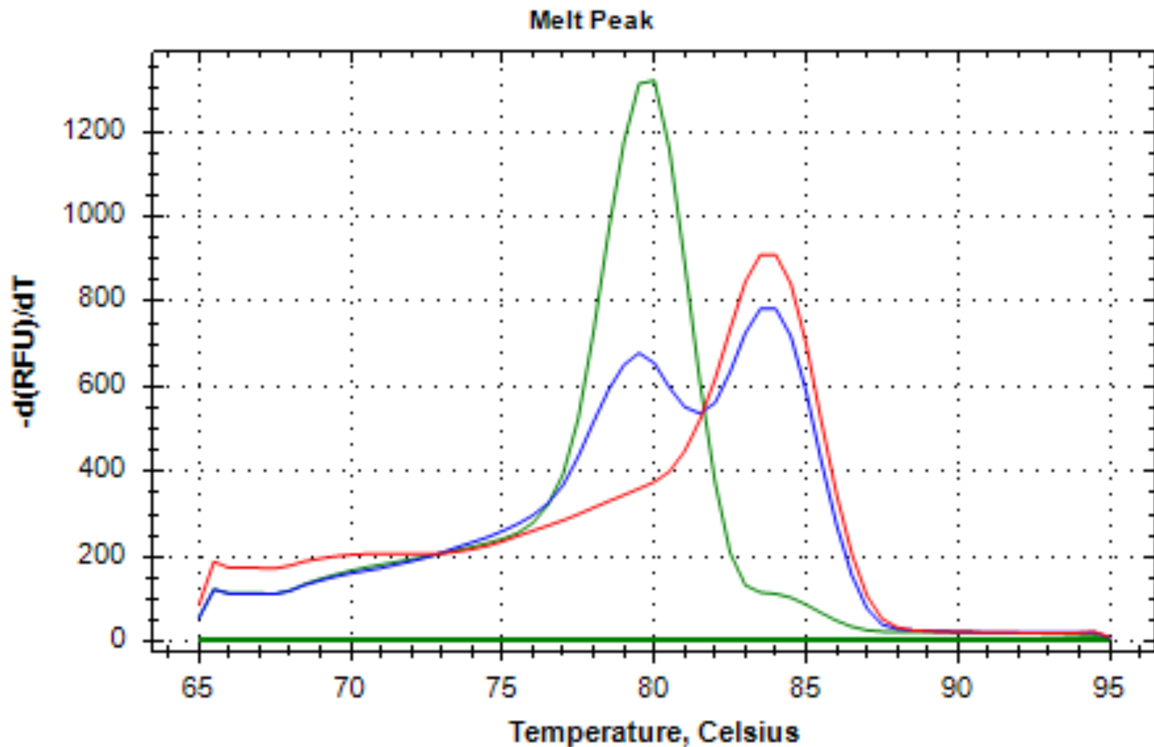


Figure 3.5: Melting curve peaks of the controls used to optimize A*S*-PCR. Green peak = phenylalanine (F/F) homozygote, susceptible phenotype (ATMNJ95 $F_{17}$ Female #17). Red peak = cysteine (C/C) homozygote, resistant phenotype (C1534_seq). Blue peaks = heterozygote (F/C) (mix of ATMNJ95 $F_{17}$ Female #17 + C1534_seq).

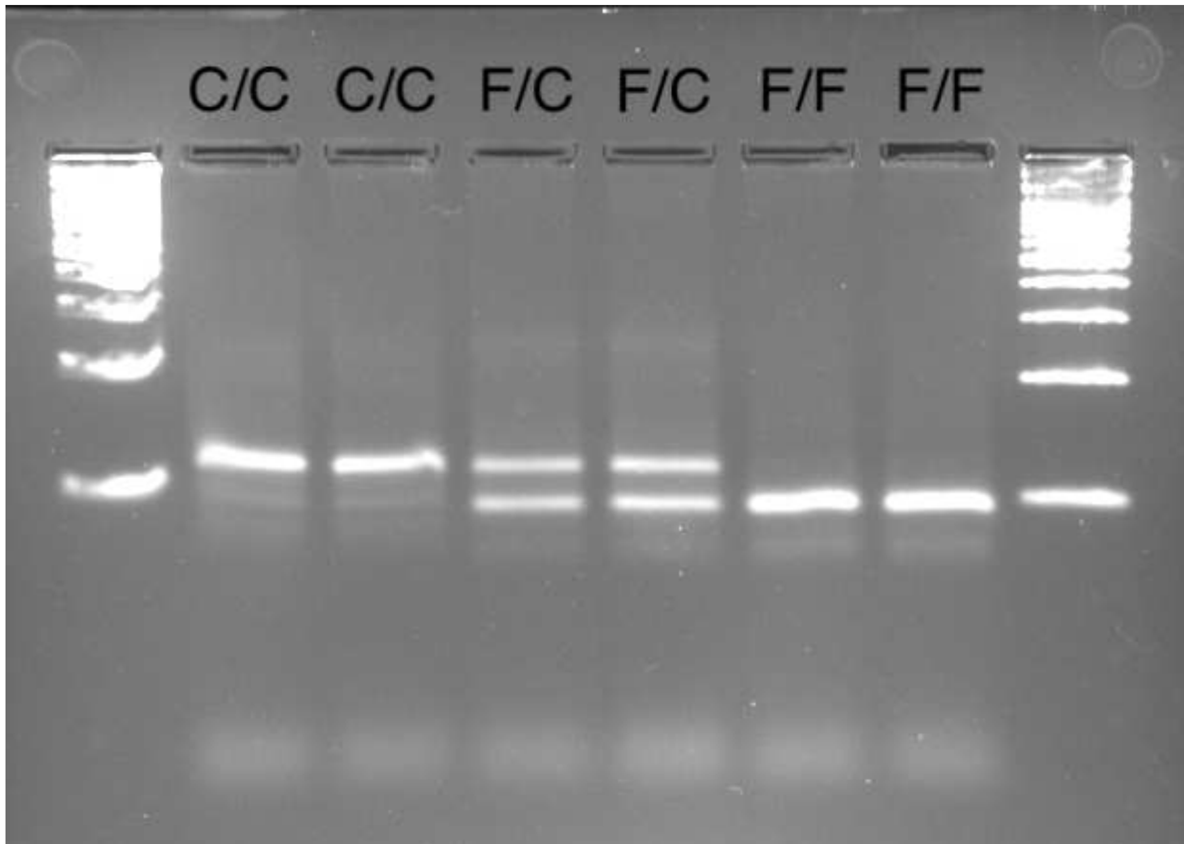


Figure 3.6: Agarose gel (3.5%) with the PCR products of the three controls used to optimize the A*S*-PCR (C/C, F/C and F/F) in duplicate. We used 100 bp ladders in the first and last wells. Recall that the C1534 products are 113 bp long, while the F1534 products are 93 bp, due to the differing GC tail lengths of the A*S*-PCR primers. The faint, small fragment bands that run past the ladders are likely primer dimers that resulted from the PCR.

Biochemical Assays

The test strains of *Ae. albopictus* that we chose to evaluate for enzyme activity were La Macha ($F_{11}$) and La Delicada ($F_{11}$) because they were the last generations to be analyzed via bottle assays. Since we had originally anticipated the La Macha $F_{11}$ generation to have the highest RR, we expected to see higher levels of enzyme activity in this group compared to La Delicada, which by the $F_{11}$ generation, had not been exposed to permethrin for 8 generations. The susceptible control strain, ATM-NJ95, was assayed for comparison.

Since we did not observe high RRs in any of our *Ae. albopictus* strains (See Chapter 2), we decided to also assess the enzyme activity of two *Ae. aegypti* strains that have been

maintained in our lab under similar conditions. The original field *Ae. aegypti* strain was collected from Merida, Yucatan, M.X. (20°57’30.08"N, 89°35’4.33"W) in 2011 and was reared in the laboratory for eight generations without exposure to insecticides. It was then divided into two separate groups. One group, Vergel-S, was maintained continuously in the laboratory without insecticide exposure. The other group, Vergel-R, was selected with permethrin using concentrations that would kill about 30-50% of the mosquitoes (concentrations ranged from 10-25 µg/bottle depending on the generation) at the $F_8$, $F_{11}$, $F_{13}$, $F_{17}$ and $F_{23}$ generations. Females from the $F_{26}$ generation from both groups were assayed for enzyme activity and compared to the *Ae. albopictus* strains. We also tested a known susceptible *Ae. aegypti* lab strain, New Orleans, for comparison. The $LC_{50}$s of the Vergel-R and Vergel-S groups (unpublished data) roughly estimate the two groups to be approximately 38 times and 2 times more resistant, respectively, than the New Orleans strain. Additionally, the I1016 *kdr* mutation (DIIS6 of VGSC, Figure 3.1) has been detected in both test strains at the $F_{27}$ generation, with frequencies of 0.88 for Vergel-R and 0.18 for Vergel-S (unpublished data). Based on the data for these two strains, we hypothesized that they would exhibit higher enzymatic activity, at least in the enzymes that contribute to metabolic pyrethroid resistance, compared to our *Ae. albopictus* strains. Moreover, we also hypothesized that the Vergel-R would have comparatively higher enzyme activity than the Vergel-S and New Orleans *Ae. aegypti* strains, since it has been selected with permethrin for multiple generations and has the highest frequency of the *kdr* mutation, I1016.

The biochemical assay protocol was modified from Valle et al. (2006). Non blood-fed female mosquitoes were collected and frozen for the biochemical assays at 4 d.p.e. and were kept at -80°C until tested. Females were kept on ice and homogenized with 300 µL of autoclaved distilled and deionized water ($ddH_2O$). Homogenates from 40 mosquitoes of the test strain and 5

mosquitoes from the control strain were transferred to a 96 well plate. From this plate, the homogenates were transferred in duplicate to separate plates for each enzymatic assay. Every assay directly quantifies the enzyme activity within individual mosquitoes, except for the mixed function oxidase (MFO) assay, which indirectly estimates cytochrome P450 (P450s) activity from heme content within each mosquito, since P450s are primarily associated with heme in non-blood-fed mosquitoes (Hemingway 1998). Enzyme absorbance values of each mosquito were measured using Bio-Rad Benchmark Plus™ microplate spectrophotometer and Microplate Manager 5.2.1 software.

For the MFO assay, 3,3',5,5'-tetramethyl-benzidine dihydrochloride (TBMZ) was used as a substrate. The MFO plate was prepared by mixing 20 μL of homogenate supernatant from each mosquito with 90 mM potassium phosphate buffer (pH 7.2), 0.03% TMBZ/250 mM sodium acetate buffer (pH 5.0), and 3% hydrogen peroxide ($H_2O_2$). Bovine heart cytochrome C (0.01 mg/ml in 250 mM sodium acetate, pH 5.0) and 90 mM potassium phosphate buffer (pH 5.0) were used as positive and negative controls, respectively, and were tested in triplicate. The plate was incubated at room temperature (in an incubator set to 23°C) for 90 min prior to reading the absorbance at 650 nm.

The acetylcholinesterase (AChE) assay was analyzed by using two plates; one was to measure normal AChE activity and the other was used to measure inhibited acetylcholinesterase activity (iAChE). Each plate was loaded with 25 μL of homogenate supernatant and mixed with 1% Triton X in 100 mM phosphate buffer (pH 7.8), 10 mM 5,5'- dithiobis 2-nitrobenzoic acid (DTNB) in 100 mM Sodium phosphate buffer (pH 7.0). The AChE plate was also mixed with 10 mM acetylcholine iodide, while the iAChE plate was mixed with 10mM acetylcholine iodide and 100 mM propoxur in acetone. The six blank wells were filled with 25 uL of $ddH_2O$ and were

used as negative controls. After incubating the plates at room temperature for 60 min, the absorbencies were read at 405 nm.

The remaining homogenates in the original plate were centrifuged prior to being transferred in duplicate to separate plates for the following assays: alpha-esterases (α-EST), beta-esterases (β-EST), p-nitrophenyl acetate esterases (PNPA), glutathione *S*-transferases (GST) and proteins (PTN). The α-EST and β-EST plates were both loaded with 10μL of mosquito supernatant, and either α- or β- naphthyl acetate/sodium phosphate as the substrate (30 mM α- or β- naphthyl acetate in 20 mM sodium phosphate buffer, pH 7.2), respectively, and were then incubated at room temperature for 15 min. Afterwards, 0.3% Fast blue B in 3.5% sodium dodecyl sulfate (SDS) was added to each plate, and then they were incubated again for 5 min. dd$H_2O$ was used as a negative control and either 3.5 nM/μL of α- or β-naphthol were used for the positive controls of the α- or β-EST plates, respectively. Absorbencies were read at 570 nm for each plate.

Mosquito homogenates were also transferred to the PNPA plate (10 μL in duplicate) and were mixed with the substrate, 1 mM p-nitrophenyl acetate in 50 mM sodium phosphate buffer, pH 7.4. Again, dd$H_2O$ was used in the negative control wells. Immediately upon mixing the substrate solution with the supernatants, the absorbencies were read at 405 nm every 15 sec for 2 min.

GST activity was measured by mixing 15 μL of homogenate with a substrate mixture of reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB), which consisted of 9.5 mM GSH in 100 mM potassium phosphate buffer, pH 6.5/1 mM CDNB in methanol. dd$H_2O$ was used for the negative controls. The absorbencies were read at 340 nm immediately after the

homogenates were mixed with the substrate solution and were read at 0, 10, and 20 min timepoints.

The amount of PTN was measured for each mosquito and used to quantify all other enzyme measurements relative to mosquito body size. PTN was assayed by mixing 10 μL of homogenate with a 1:4 mixture of Bio-Rad reactive (Bio-Rad, Hercules, CA) and $ddH_2O$. 1 mg/mL Bovine Serum Albumin (BSA) and $ddH_2O$ were used as positive and negative controls, respectively. The plate was incubated for 3 min at room temperature before the absorbencies were read at 620 nm.

Statistical Analysis of Biochemical Assays

Enzyme activity was calculated from the measured absorbance values using Microsoft Excel, following the calculations from Valle et al. (2006). We used R 3.6.0 software to calculate one-way analysis of variance (ANOVA) and pair-wise t-tests (with Bonferroni adjustment) and to graph box plots to compare differences in mean enzyme activity between strains. The Bonferroni corrected p-value was 0.003 [0.05 (original p-value)/15 (# of comparisons)], so there was a significant difference between groups when $p < 3e-3$. The absorbance values of the MFO, α-EST, β-EST and PTN were converted to appropriate enzymatic activity measurements by using standard absorbance curves with known quantities of cytochrome C, α-naphthol, β-naphthol and BSA, respectively (PNPA and GST assays cannot be converted this way because there are no standard substrates available to create standard absorbance curves).

**Results**

Sequencing and Genotyping *kdr* Mutations

We did not detect the C1534 mutation in any of the mosquitoes sampled from the eleven sites in Mexico, or from any of the mosquitoes in either of our pooled Mexico strains (La Macha $F_{11}$ survivors, La Delicada $F_{11}$). In other words, out of the 700 *Ae. albopictus* mosquitoes we genotyped (including the ATMNJ95 strain), all of the mosquitoes had the homozygous susceptible genotype (F1534F).

Biochemical Assays

Overall there were significant differences between the tested strains of mosquitoes for every enzyme assay, as the ANOVA p-values were all $< 0.05$. The p-values that are reported in the following paragraphs are all from the pair-wise t-test analyses (with Bonferroni adjustment) between individual strains.

With the addition of propoxur, we observed that AChE was least inhibited in the Vergel-R strain, which had an average inhibition of 82.56%, and was significantly lower than all of the other strains except ATMNJ95, which was inhibited by 86.21% ($p = 2.6e-2$). The ATMNJ95 inhibition was also similar to the La Macha strain, which was inhibited by 87.53%. AChE was most inhibited in the New Orleans, Vergel-S and La Delicada strains, ranging from 90.21 – 91.82% inhibition (Figure 3.7, Table 3.2).

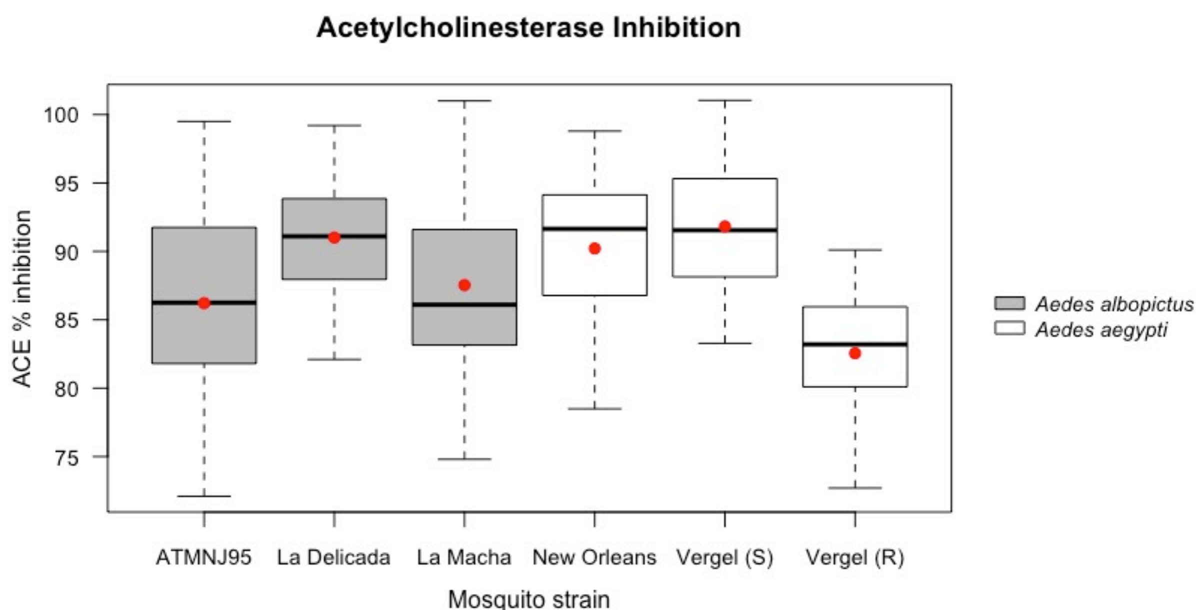


Figure 3.7: Boxplots comparing percent of acetylcholinesterase (ACE) inhibition between *Ae. albopictus* and *Ae. aegypti* strains. The boxes represent the interquartile ranges (i.e. range between the 25th and 75th percentiles) and the whiskers that extend from the boxes represent the min and max observed values (excluding outliers). Red dots denote the means of each strain and the black lines within the boxes are the medians.

Table 3.2: Corresponding p-values (p < 3e-3 meant a significant difference between strains) from pair-wise t-test analysis (with Bonferroni adjustment) of Acetylcholine activity.

| | ATMNJ95 | La Delicada | La Macha | New Orleans | Vergel (R) |
|---|---|---|---|---|---|
| La Delicada | 6.9e-4 | - | - | - | - |
| La Macha | 1 | 4.36e-2 | - | - | - |
| New Orleans | 9.47e-3 | 1 | 3.17e-1 | - | - |
| Vergel (R) | 2.62e-2 | 6e-11 | 3.6e-4 | 3.4e-9 | - |
| Vergel (S) | 3.3e-5 | 1 | 3.81e-3 | 1 | 7.3e-13 |

The activity of P450s was greater in the *Ae. albopictus* strains relative to their counterpart *Ae. aegypti* strains (i.e. the control *Ae. albopictus* had higher P450 activity compared to the control *Ae. aegypti* strain, and so on), with all p-values < 3e-3. There was also a similar pattern observed when comparing the control strains to the test strains of both species. The control *Ae.*

*albopictus* strain, ATMNJ95 had significantly lower levels of P450 activity (0.17 µg cit/ptn on average) compared to the La Macha and La Delicada strains, which both had 0.20 µg cit/ptn on average ($p < 2e-16$). The control *Ae. aegypti* strain, New Orleans, had 0.13 µg cit/ptn of P450 activity compared to the test strains, Vergel-R ($p = 1.4e-6$) and Vergel-S ($p = 2.5e-7$), which both had averages of 0.15 µg cit/ptn (Figure 3.8, Table 3.3).

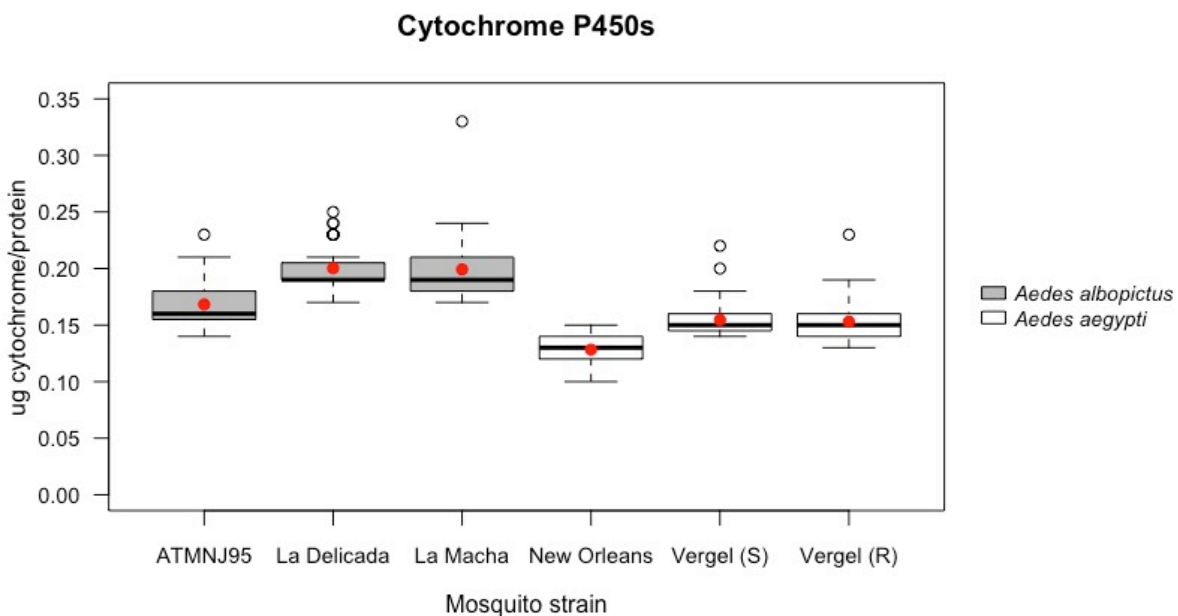


Figure 3.8: Boxplots comparing amount of cytochrome P450s between *Ae. albopictus* and *Ae. aegypti* strains. The boxes represent the interquartile ranges (i.e. range between the 25$^{th}$ and 75$^{th}$ percentiles) and the whiskers that extend from the boxes represent the min and max observed values (excluding outliers). Red dots denote the means of each strain and the black lines within the boxes are the medians.

Table 3.3: Corresponding p-values ($p < 3e-3$ meant a significant difference between strains) from pair-wise t-test analysis (with Bonferroni adjustment) of cytochrome P450 activity.

| | ATMNJ95 | La Delicada | La Macha | New Orleans | Vergel (R) |
|---|---|---|---|---|---|
| La Delicada | 1.9e-10 | - | - | - | - |
| La Macha | 7.1e-10 | 1 | - | - | - |
| New Orleans | 2.2e-15 | < 2e-16 | < 2e-16 | - | - |
| Vergel (R) | 1.2e-2 | < 2e-16 | < 2e-16 | 1.4e-6 | - |
| Vergel (S) | 3.7e-2 | < 2e-16 | < 2e-16 | 2.5e-7 | 1 |

Alpha-esterase activity was significantly higher in the *Ae. albopictus* strains compared to the *Ae. aegypti* strains (p values were all < 3e-3) and within the species there were no significant differences between strains (p values > 3e-3), see Table 3.4. Between the two species there were no distinct patterns among the counterpart strain types. Out of all six strains, La Delicada had the highest alpha-esterase activity (11.75 nmol naphthol/mg ptn/min) and New Orleans had the lowest activity (8.26 nmol naphthol/mg ptn/min) (Figure 3.9, Table 3.4).

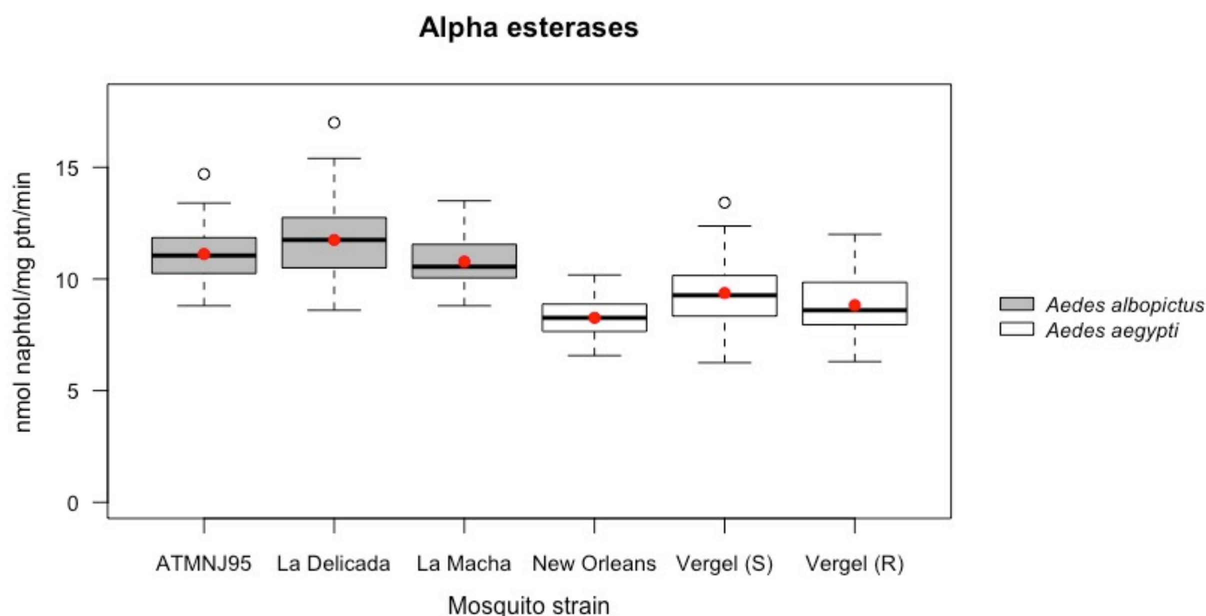


Figure 3.9: Boxplots comparing amount of alpha-esterase activity between *Ae. albopictus* and *Ae. aegypti* strains. The boxes represent the interquartile ranges (i.e. range between the 25$^{th}$ and 75$^{th}$ percentiles) and the whiskers that extend from the boxes represent the min and max observed values (excluding outliers). Red dots denote the means of each strain and the black lines within the boxes are the medians.

Table 3.4: Corresponding p-values ($p < 3e-3$meant a significant difference between strains) from pair-wise t-test analysis (with Bonferroni adjustment) of alpha-esterase activity.

| | ATMNJ95 | La Delicada | La Macha | New Orleans | Vergel (R) |
|---|---|---|---|---|---|
| La Delicada | 5.17e-1 | - | - | - | - |
| La Macha | 1 | 1.88e-2 | - | - | - |
| New Orleans | < 2e-16 | < 2e-16 | 3.3e-14 | - | - |
| Vergel (R) | 4.7e-12 | < 2e-16 | 4.3e-9 | 8.57e-1 | - |
| Vergel (S) | 1.9e-7 | 7.4e-13 | 5.2e-5 | 3.4e-3 | 1 |

Beta-esterase activity was observably lower than alpha-esterase activity for both species. Again, there were no distinct patterns observed between strain types. ATMNJ95 and La Delicada had the highest beta-esterase activity (8.10 and 8.19 nmol naphthol/mg ptn/min, respectively),

while New Orleans and Vergel-R had the lowest activity (4.19 and 4.45 nmol naphthol/mg ptn/min, respectively) out of all the assayed strains (Figure 3.10, Table 3.5).

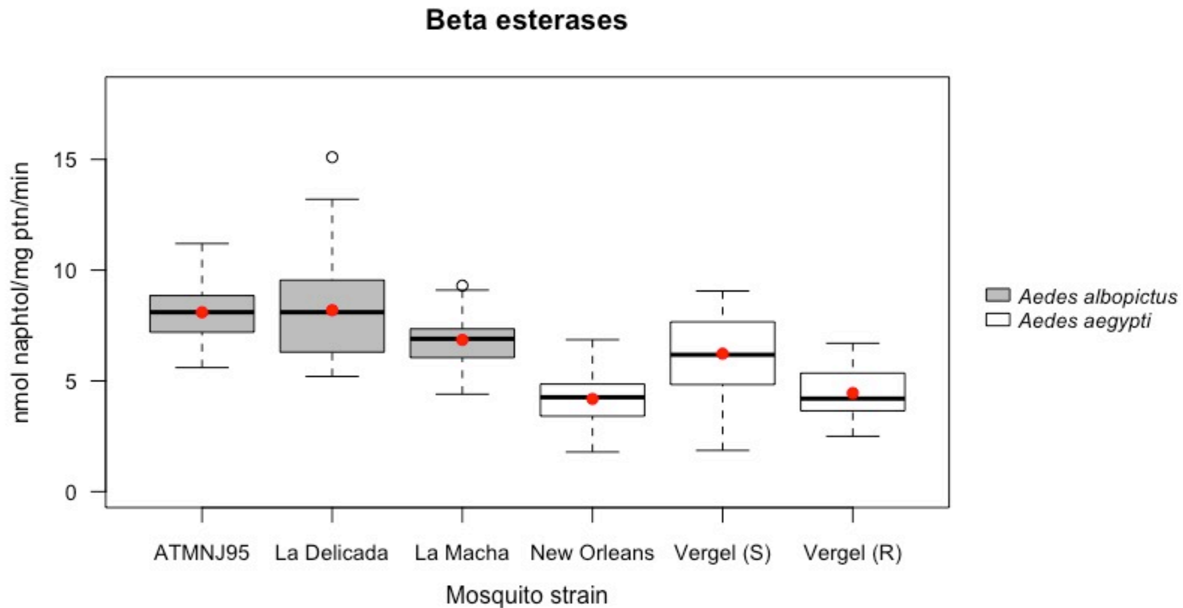


Figure 3.10: Boxplots comparing amount of beta-esterase activity between *Ae. albopictus* and *Ae. aegypti* strains. The boxes represent the interquartile ranges (i.e. range between the 25th and 75th percentiles) and the whiskers that extend from the boxes represent the min and max observed values (excluding outliers). Red dots denote the means of each strain and the black lines within the boxes are the medians.

Table 3.5: Corresponding p-values ($p < 3e-3$ meant a significant difference between strains) from pair-wise t-test analysis (with Bonferroni adjustment) of beta-esterase activity.

| | ATMNJ95 | La Delicada | La Macha | New Orleans | Vergel (R) |
|---|---|---|---|---|---|
| La Delicada | 1 | - | - | - | - |
| La Macha | 4.2e-3 | 1.5e-3 | - | - | - |
| New Orleans | < 2e-16 | < 2e-16 | 1.9e-12 | - | - |
| Vergel (R) | < 2e-16 | < 2e-16 | 2.4e-10 | 1 | - |
| Vergel (S) | 1.3e-6 | 3.3e-7 | 1 | 9.1e-8 | 5.2e-6 |

There was also no distinguishable pattern observed among the six strains when comparing the different levels of GST activity. Out of all the strains, New Orleans had the highest mean value of GST activity (1.00 mmol/mg ptn/min) while La Macha had the lowest (0.68 mmol/mg ptn/min) (Figure 3.11, Table 3.6).

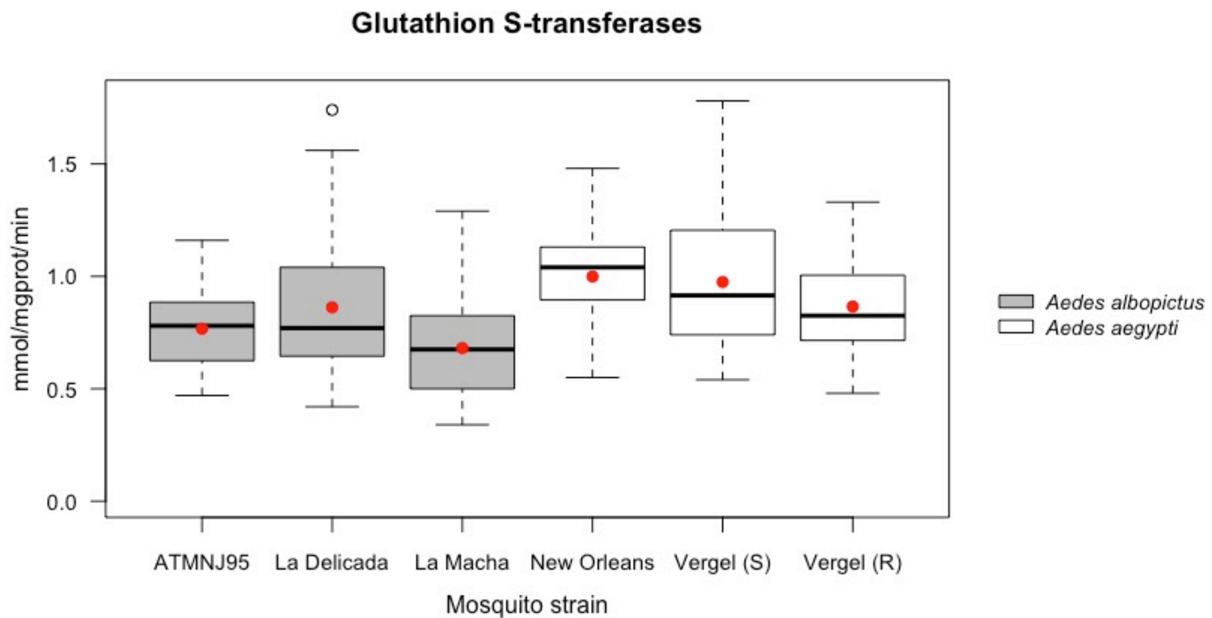


Figure 3.11: Boxplots comparing amount of glutathione *S*-transferase activity between *Ae. albopictus* and *Ae. aegypti* strains. The boxes represent the interquartile ranges (i.e. range between the 25th and 75th percentiles) and the whiskers that extend from the boxes represent the min and max observed values (excluding outliers). Red dots denote the means of each strain and the black lines within the boxes are the medians.

Table 3.6: Corresponding p-values ($p < 3e-3$ meant a significant difference between strains) from pair-wise t-test analysis (with Bonferroni adjustment) of glutathione *S*-transferase activity.

| | ATMNJ95 | La Delicada | La Macha | New Orleans | Vergel (R) |
|---|---|---|---|---|---|
| La Delicada | 1 | - | - | - | - |
| La Macha | 1 | 2.33e-2 | - | - | - |
| New Orleans | 8.4e-4 | 2.42e-1 | 7.9e-7 | - | - |
| Vergel (R) | 1 | 1 | 1.89e-2 | 2.85e-1 | - |
| Vergel (S) | 4.41e-3 | 7.08e-1 | 6.6e-6 | 1 | 8.17e-1 |

The PNPA activity levels also did not seem to exhibit any particular patterns among the different mosquito strains. The highest average of PNPA activity was observed in Vergel-R (2.88 Δ ABS/mg ptn/min) and the lowest was observed in La Macha (1.76 Δ ABS/mg ptn/min) (Figure 3.12, Table 3.7). Refer to Table A.3.2 for a complete list of means and standard errors from each enzyme assay.

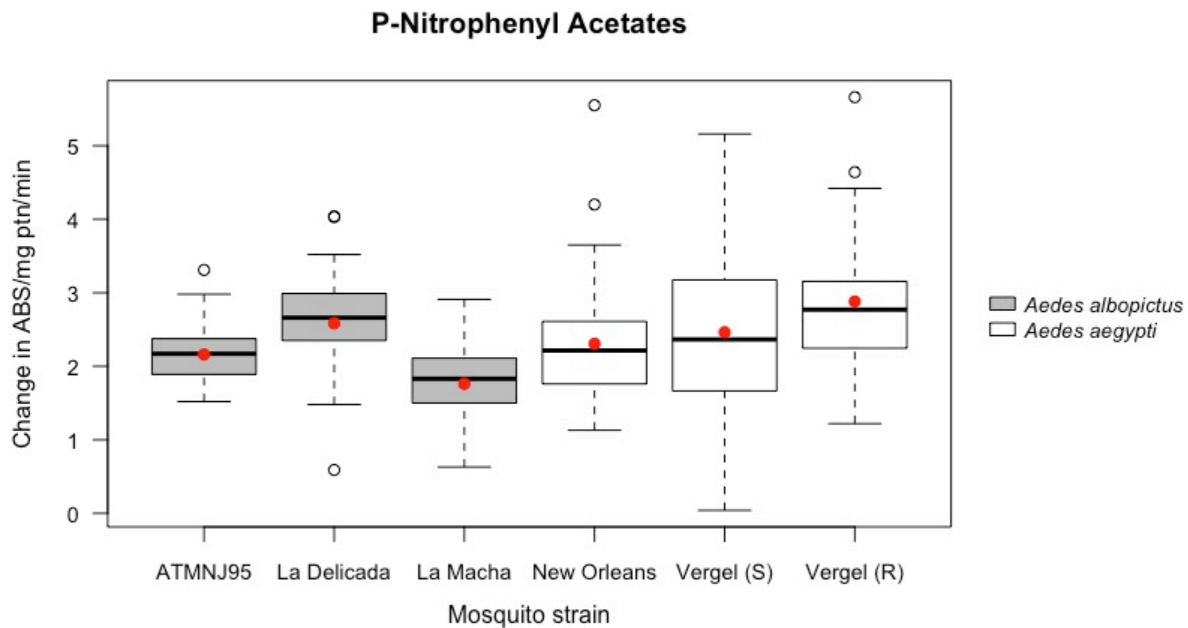


Figure 3.12: Boxplots comparing amount of p-nitrophenyl acetate esterase activity between *Ae. albopictus* and *Ae. aegypti* strains. The boxes represent the interquartile ranges (i.e. range between the 25th and 75th percentiles) and the whiskers that extend from the boxes represent the min and max observed values (excluding outliers). Red dots denote the means of each strain and the black lines within the boxes are the medians.

Table 3.7: Corresponding p-values ($p < 3e-3$ meant a significant difference between strains) from pair-wise t-test analysis (with Bonferroni adjustment) of p-nitrophenyl acetate esterase activity.

| | ATMNJ95 | La Delicada | La Macha | New Orleans | Vergel (R) |
|---|---|---|---|---|---|
| La Delicada | 2.23e-1 | - | - | - | - |
| La Macha | 3.44e-1 | 5.9e-5 | - | - | - |
| New Orleans | 1 | 1 | 2.89e-2 | - | - |
| Vergel (R) | 6.9e-4 | 1 | 1.2e-8 | 1.63e-2 | - |
| Vergel (S) | 1 | 1 | 1.1e-3 | 1 | 2.53e-1 |

**Discussion**

Sequencing and Genotyping *kdr* Mutations

At the start of this project, when we originally hypothesized that *Ae. albopictus* from our study sites would exhibit resistance to permethrin, we had also anticipated that we would find

*kdr* mutations that are known to confer resistance in populations of mosquitoes exhibiting a resistant phenotype to permethrin. However, results from the bottle assay data (Chapter 2) indicated that *Ae. albopictus* has low levels of resistance relative to our susceptible control strain, therefore, we hypothesized that it would be unlikely to observe any mutations in the 1534 site of the VGSC. Even though all of our field strains exhibited low levels of resistance, at the start of this project we also anticipated the possibility that we would select for *kdr* mutations in our La Macha strain. While the La Macha strain initially seemed as if it was becoming more resistant with continued selection, in the end we were surprised to see that the RR significantly dropped in our last selected generation (Chapter 2). In retrospect, it probably would have been better to screen the La Macha $F_9$ generation, since it ended up having the highest RR compared to the other generations of La Macha, however, since we did not anticipate these results, we did not collect females from this generation for genotyping.

Despite these setbacks with our bottle assays, we were still surprised by the genotyping results since the frequency of the F1534C mutation has increased over the last 16 years (García et al. 2009) and has reached fixation in multiple populations of *Ae. aegypti* from Southern Mexico (Vera-Maloof et al. 2015). A possible explanation for this interspecies difference could be that *Ae. aegypti* has been exposed more frequently to insecticides due to their anthropogenic behavior. For example, a recent study in Brazil found that in a municipal urban park, *Ae. aegypti* mosquitoes were primarily found near the peripheral edges of the park, which was in close proximity to humans, while *Ae. albopictus* were primarily in the center of the park where there was a higher abundance of vegetation (Heinisch et al. 2019). While Heinisch et al. (2019) did not assess the levels of resistance in their mosquitoes, they highlight the importance of considering the ecology and dispersal patterns of different vector species when implementing control

strategies such as insecticides, since urban areas are primarily targeted and species like *Ae. albopictus* might be evading such control measures due to their dispersal patterns. Further studies are needed to confirm this.

Another explanation for the higher frequencies of *kdr* mutations in *Ae. aegypti* could be that this species has been present in the Americas for a much longer time compared to *Ae. albopictus*, and therefore experienced more selection pressure over a longer period of time due to the *Ae. aegypti* eradication efforts that began in the 1940s. Only three mutations of the VGSC have been reported in *Ae. albopictus* from the Americas. The I1011M/V mutations were detected in *Ae. albopictus* from Haiti (McAllister et al. 2012) and the F1534C was reported in *Ae. albopictus* from Brazil (Aguirre-Obando et al. 2017). Interestingly, the majority of the mutations that have currently been reported in *Ae. albopictus* have occurred in populations from Asia (Kasai et al. 2011, 2019, Chen et al. 2016, Xu et al. 2016, Gao et al. 2018, Li et al. 2018, Zhou et al. 2019), which is where *Ae. albopictus* originated from. Refer back to the Chapter 2 discussion for specific details on the different phenotypic resistance patterns that have been observed between *Ae. albopictus* in native and non-native regions.

Biochemical Assays

Out of all the enzymes that we assayed, the P450s were the only enzyme that seemed to have a distinct pattern among the strains we tested. The three *Ae. albopictus* strains all had significantly higher P450 activity compared to the three *Ae. aegypti* counterpart strains (i.e. control strains, non-selected test strains and permethrin-selected test strains). Additionally, within each species, the three strain types all exhibited similar patterns. Among the *Ae. albopictus* strains, the control strain, ATMNJ95, had significantly lower levels of P450 activity

compared to La Macha and La Delicada, which had equal amounts of activity. Within the *Ae. aegypti* strains, New Orleans had significantly lower P450 activity compared to the Vergel strains, which had equal activity levels.

However, from these results we cannot clearly determine if P450s have a mechanistic role in resistance in the *Ae. albopictus* we tested. Further studies using assays with synergists, such as piperonyl butoxide (PBO), which is an inhibitor of P450s (Liu 2015), would help confirm if P450s are actually conferring resistance in *Ae. albopictus*. Outside of the P450s, we did not observe any distinct patterns or elevated enzyme activity as we had expected to see in our resistant strains. For example, the Vergel-R strain, which has been selected with high concentrations of permethrin (10-25 μg/bottle) over multiple generations, did not have the highest levels of enzyme activity as we originally hypothesized it would. This was particularly surprising because among our tested strains, Vergel-R was the most resistant strain we tested, based on the high $LC_{50}$ values we selected it with and the 0.88 frequency of the I1016 mutation in the $F_{27}$ generation (unpublished data). We might speculate that the reason we did not observe higher enzymatic activity in Vergel-R could be because it has a high frequency of the I1016 mutation (and possibly other *kdr* mutations), which thereby confer most of the phenotypic resistance to permethrin by means of altering the VGSC, and possibly making enzyme detoxification of the insecticide less essential. We would have to confirm this hypothesis with future testing. However, the one difference in enzymatic activity we did observe among the Vergel-R strain was that it had significantly lower inhibition of AChE compared to all of the other strains. This might suggest that Vergel-R has some level of resistance to organophosphates or carbamates, since these insecticides target AChE, however we did not confirm this from bottle assays, so this is only speculation. Future assays would also need to be done in order to test this.

Unfortunately, the lack of observable or distinguishable patterns among any of our other enzyme analyses could also be simply due to the limitations of biochemical assays. For instance, we are observing the enzyme activity based on the presence of enzymes in mosquitoes that are dead (at the point they are frozen), and within mosquitoes that are not being exposed to insecticides. This leaves us with the ability to analyze the total enzyme activity present in the mosquito at the time of the biochemical assays. As previously mentioned at the beginning of this chapter, these enzymes are naturally present in mosquitoes and serve other purposes besides just detoxifying insecticides. It is possible the enzyme activity we are observing is just a baseline activity present in the mosquitoes at the moment they died. Although it would be ideal, we unfortunately cannot directly measure and compare the levels of enzymatic activity in mosquitoes before and after exposure to an insecticide to see if increased enzyme detoxification occurs at higher levels in resistant mosquitoes compared to susceptible ones. Another downfall of the biochemical assay is that it measures total enzymatic activity. For example, with the MFO assay, we were able to measure the total activity of all P450s present in our mosquito strains, but that does not mean that all of the P450s are contributing to insecticide resistance. For instance, *Ae. aegypti* has around 160 genes that encode P450s (Strode et al. 2008) but not all of them have been implicated in resistance. Regarding metabolic resistance in *Ae. albopictus*, one study specifically found that the CYP6P12 gene was upregulated in resistant *Ae. albopictus* from Malaysia in the absence of *kdr* mutations (Ishak et al. 2016). Future studies similar to the aforementioned one, would be beneficial to conduct on the *Ae. albopictus* from our collection sites in order to more clearly understand if specific P450 genes, or other enzyme detoxifying genes, are conferring metabolic resistance in the species.

Overall, we were not able to detect or determine if *kdr* mutations or metabolic enzymes play a role in resistance in the *Ae. albopictus* strains we tested, but this data does correlate with the low RRs we observed in our bottle bioassays (Chapter 2). Future monitoring of resistance and resistance-conferring mechanisms should be conducted in our study sites in order to ensure that permethrin, and other insecticides, can continue to be used as effect methods for controlling *Ae. albopictus* populations and the diseases they transmit.

# CHAPTER 4 – SUMMARY AND FUTURE DIRECTIONS

## Summary

Prior to beginning this study, we expected to see moderate to high levels of permethrin resistance and to identify possible *kdr* mutations or enzymes imparting resistance in our field and artificially selected strains of *Ae. albopictus*. However, our results did not support our initial assumptions. Low levels of permethrin resistance were observed in our populations of *Ae. albopictus* from Mexico (M.X.) and Texas relative to a susceptible control strain. The G (Col. 5 Febrero) and WCC strains had the highest levels of resistance observed in the field populations from M.X. and Texas, respectively. It could be argued that our resistance ratios (RRs) are too low to confirm resistance to permethrin. However, aside from the Puerto Madero strain, all the assayed *Ae. albopictus* strains had RRs and 95% HDI greater than 1, indicating that they were less sensitive to permethrin than our ATMNJ95 control strain. It is possible that the RRs are so low because we are observing the initial stages of permethrin resistance in these *Ae. albopictus* strains. Alternatively, we might also be observing the opposite situation, in which resistance in our *Ae. albopictus* field strains is lower than it once was, due to adaptations of mosquito control and resistance monitoring and management.

We also attempted to increase resistance by recurrent selection of the La Macha strain over several generations with a range of permethrin concentrations. The RR of the La Macha strain was significantly higher in the $F_9$ generation compared to the $F_5$ generation, but there was an unexpected significant decrease of the RR in the $F_{11}$ generation. We were also surprised to find that the RR of the La Macha $F_{11}$ was significantly lower than the RR of La Delicada $F_{11}$, the non-selected counterpart strain. There are several possible explanations as to why the RRs of La

Macha dropped after continued selection with permethrin. It is possible that while we were attempting to select for permethrin resistance, more inbreeding occurred and increased the number of individuals homozygous for lethal or deleterious mutations, thereby making them more susceptible to permethrin. Another explanation could be that we did not select over enough generations to observe an increase in resistance within our population. This could be due to heterozygous (*kdr*) individuals diluting the gene pool in La Macha, thereby allowing the susceptible phenotype to dominate the population. This explanation was also suggested by Selvi et al (2010), whom also observed a decline in resistance when attempting to select *Ae. albopictus* in the laboratory. One other possible reason we observed this unexpected change in RRs between the La Macha and La Delicada strains, could simply be due to random variation. For example, if the RR of La Macha happened to be much higher, let us say around 20, then by comparison, a drop in the RR by factor of 1 in the next generation (RR being 19) would not seem so significant. Since the RRs were low for all of our tested populations, it is possible that variation between strains could seem significant, even though it might not be.

We also searched for the presence of *kdr* mutations and elevated enzyme activity to understand if either of these mechanisms were causing the low levels of resistance observed in the *Ae. albopictus* populations tested in this study. Specifically, we screened for the F1534C mutation of the voltage gated sodium channel (VGSC) gene in *Ae. albopictus* from the eleven collection sites in M.X., and from the La Macha, La Delicada, and ATMNJ95 strains. In the 700 individual mosquitoes genotyped, all of the mosquitoes were homozygous susceptible for phenylalanine at the 1534 site (TTC codon). Since the F1534C mutation normally imparts strong resistance to permethrin, these results are consistent with the fact that our populations of *Ae. albopictus* had low levels of resistance. It is possible that the mutations are present in our

populations but are in such low frequency that we were unable to detect them. It is also possible that other *kdr* mutations are present in the populations we sampled, but we were unable to screen for these mutations in this study.

There were no observable patterns of elevated enzyme activity among most of the metabolic enzymes tested (acetylcholinesterases, alpha- and beta- esterases, glutathione *S*-transferases, p-nitrophenyl acetates) in the laboratory strains of *Ae. albopictus* or *Ae. aegypti*. We did observe significantly higher activity of P450s in the La Macha and La Delicada strains compared to the ATMNJ95 and all three *Ae. aegypti* strains. There was a similar pattern of P450 activity within each species, with the two susceptible strains (ATMNJ95 and New Orleans) having significantly lower P450 activity compared to the non-selected (La Delicada and Vergel-S) and permethrin-selected (La Macha and Vergel-R) strains. The selected and non-selected strains had equal amounts of P450 activity within each species, but with the *Ae. albopictus* strains (La Delicada and La Macha) having significantly higher P450 activity than their *Ae. aegypti* counterparts. However, from these results we still could not determine if P450s have a mechanistic role in permethrin resistance within our tested mosquito strains.

Overall, there are low levels of permethrin resistance in *Ae. albopictus* from the sites we sampled in southern M.X. and Texas compared to our susceptible control strain. We did not find any mutations or elevated enzyme activity in our *Ae. albopictus* mosquitoes that have been previously associated with pyrethroid resistance in other mosquitoes. From this study we conclude that even with the low levels of resistance detected in *Ae. albopictus*, they are still susceptible to the concentrations of permethrin that are used to control *Aedes* mosquitoes. However, it is important to continue to monitor resistance and resistance-conferring mechanisms

in *Ae. albopictus* to ensure that they can be continuously controlled with permethrin and other insecticides in order to prevent the spread of arboviral diseases.

**Resistance Monitoring and Management: Limitations and Areas Needing Improvement**

This study has brought attention to several limitations and areas needing improvement in the evaluation of insecticide resistance in mosquito populations. First, it is difficult to directly compare across studies the levels of resistance observed in *Ae. albopictus* mosquitoes, even within the same regions or cities, due to the different methodologies that are used for testing resistance levels. Some groups followed WHO tube assay protocols, while others used the CDC bottle assay procedures or even direct topical application. Even with these commonly used methodologies, there are different ways to interpret the data. Some groups compared $LC_{50}$s/$LD_{50}$s and RRs, while others evaluated resistance based on diagnostic concentrations established by WHO or CDC protocols. This variation in methodology and data interpretation can make it difficult to compare results among multiple studies. Several other labs have also acknowledged this difficulty in comparing levels of insecticide resistance due to the various methods used (Ranson et al. 2010, Vontas et al. 2012, Pichler et al. 2018).

Consistency of methodology is not the only issue when it comes to comparing results of multiple studies that have investigated resistance and resistance-conferring mechanisms in mosquitoes. While many studies have found the presence of *kdr* mutations (resistance conferring mutations) in the VGSC of mosquitoes, not all studies have actually evaluated the physical resistance of the populations from which these mutations were found, making it difficult to interpret if the mutations are actually conferring resistance. For example, Kasai et al. (2011) and Aguierre-Obando, Martins, and Navarro-Silva (2017) reported F1534C mutations in the *Ae.*

*albopictus* they screened and suggested that they were conferring resistance due to the heavy use of pyrethroids that were being sprayed in the areas, however the mosquitoes were not tested for resistance to pyrethroids. While the F1534C mutation functionally reduces the VGSC sensitivity to pyrethroids in *Ae. aegypti* (Du et al. 2013) it cannot be assumed that this mutation is equally affecting resistance in *Ae. albopictus*. In order to more accurately confirm and report if mutations are associated with resistance, it is important that all studies searching for possible *kdr* mutations in *Ae. albopictus*, and other insect species, also test the physical levels of resistance in mosquito populations to determine if there is statistical association between genotype and phenotype.

Another issue regarding published studies on insecticide resistance was addressed in a review by Ranson et al. (2010), in which they summarized multiple studies on resistance in *Ae. albopictus* and *Ae. aegypti* to multiple classes of insecticides and suggested that there is likely a bias towards publishing positive results of resistance over negative results. While our data show a possible or low level of resistance in our populations, some might suggest our results could be interpreted as “negative” results, since the RRs were low and we were unable to detect or determine if there were underlying mechanisms of resistance. Regardless of this potential opinion, we believe there is valuable importance in reporting any levels of resistance or possible resistance conferring mechanisms (confirmed to be associated with phenotypic resistance), or lack thereof, in publications. These reports allow us to truly determine if and when resistance develops or dissipates in populations based on the current practices that are implemented in the field.

Furthermore, one of the major issues associated with this study was our inability to determine exactly what insecticides were (and are) being sprayed in the sites we collected from. The most information we could find about insecticide use in M.X. was from the annual lists that

the country is given, which indicate the potential insecticides and their respective quantities that can be used in a given year (Secretaría de Salud 2015, 2016). However, this is a list of options for insecticidal control, and it varies from state to state and city to city as to what insecticides they choose to apply, but the actual insecticides that are being used in each area are not disclosed in any documentation. In regards to the U.S., officials at state and local levels determine which insecticides should be used to control mosquitoes in their respective regions based on insecticide resistance data collected for that region (CDC 2019b). However, we also could not find what insecticides were specifically being sprayed in our collection sites from Weslaco, Texas. There are complications with reporting this information to the public, most likely due to the competitive nature of companies that sell and supply insecticides. However, if public health and resistance management strategies are to be prioritized, then there is a need for more transparency regarding the amount and types of insecticides being applied in regions that are being studied by researchers.

**Future Directions**

While this project has provided valuable information regarding the current status of permethrin resistance in *Ae. albopictus* from M.X. and Texas, there is still much to be learned about insecticide resistance in this species. It would be beneficial to conduct future studies to continue monitoring permethrin resistance in *Ae. albopictus* from M.X. and Texas (and other regions around the world) to see if resistance changes over time. It would also be beneficial to test if *Ae. albopictus* in these regions have currently developed resistance to any other classes of insecticides, since we were unable to evaluate this in this study. Oftentimes it is reported that pyrethroids are one of the most commonly used insecticides worldwide, but perhaps this is

changing. During the 1980s, pyrethroid use increased rapidly and comprised about 20% of the total insecticide market share by 1986 and remained around 17% of the market share through the 1990s and 2000s (Housset and Dickmann 2009). However, a more recent review found that among the top 30 insecticides used in 2015, pyrethroids only comprised about 12.5% of insecticide sales (Casida and Bryant 2017). This might suggest that pyrethroids are not as heavily used as they once were, however, this could vary from region to region.

In order to better understand the results observed in this study with artificial recurrent selection, it would be advantageous to continue to pressure *Ae. albopictus* with permethrin over multiple generations to see if resistance can be increased with long-term selection. Alternatively, it would be informative to select *Ae. albopictus* with other insecticides over multiple generations to see if resistance can be increased with insecticides that have other modes of action (e.g. organophosphates, *Bti*, IGRs, etc.). Additionally, it would be helpful to screen for the presence of *kdr* mutations in field populations in M.X. and Texas in the future to see if they can be detected and if they increase in frequency over time. Unfortunately, we were only able to screen for the cysteine mutation at the 1534 site of the VGSC in this study. It would be useful to search for other mutations, especially the F1534S and F1534L mutations, since they are associated with pyrethroid resistance in *Ae. albopictus* (Xu et al. 2016, Gao et al. 2018, Li et al. 2018). It would also be advantageous to monitor for the presence of the V1016G mutation in *Ae. albopictus*, now that it has been found in association with pyrethroid resistant *Ae. albopictus* from Vietnam and Italy (Kasai et al. 2019). The V1016G mutation has also rapidly increased in populations of *Ae. aegypti* in several Latin American countries and it has been suggested that selection for this mutation in the field occurs easily (Saavedra-Rodriguez et al. 2007, García et al. 2009, Vera-

Maloof et al. 2015), so it is possible that it could be detected in *Ae. albopictus* populations if pyrethroid resistance continues to grow.

Lastly, it would also be interesting to conduct field studies on the dispersal patterns of *Ae. albopictus* and *Ae. aegypti* to understand if there are environmental or behavioral factors that are resulting in different levels of insecticide resistance between the two species. We know that there are some shared habitat preferences between the *Ae. aegypti* and *Ae. albopictus* mosquitoes from M.X. since we collected both species from the same containers in 2016. For this study, mosquitoes were only collected from within the city of Tapachula and from several nearby rural towns. However, these areas are surrounded by dense rainforest habitats that were not sampled. It would be interesting to collect mosquitoes from these rainforest regions and compare them with collections from within and around the city to see if *Ae. albopictus* are more prevalent in the rainforest habitats. A similar study was done by Heinisch et al (2019) and they found that in an urban park in Brazil, *Ae. aegypti* were more closely associated with the periphery of the park, which was surrounded by an urban area, whereas the *Ae. albopictus* were more concentrated in the center of the park where there was more vegetation. It would be interesting to see if *Ae. albopictus* in Tapachula are more prevalent in the rainforest areas, because it could possibly explain why the species seems less resistant to permethrin compared to *Ae. aegypti*, which we would suspect to be more closely associated with the urban areas. Insecticide spraying is typically targeted at urban areas rather than rural or forested areas. This assumption would also need to be confirmed, but if it is true, it could mean that *Ae. aegypti* mosquitoes receive more selection pressure than *Ae. albopictus*, and have developed a higher resistance to insecticides. Also, if there are susceptible populations of *Ae. albopictus* being maintained in the rainforest areas surrounding Tapachula from lack of insecticide exposure, perhaps there is gene flow

providing susceptible alleles to urban populations, which might explain why we are not observing high levels of resistance in the *Ae. albopictus* in the urban collection sites from M.X.

These questions and many others should be investigated to increase our understanding on the levels of resistance and resistance-conferring mechanisms in *Ae. albopictus* in order to continuously and effectively control these mosquitoes and reduce the spread of the arboviruses they vector.

# REFERENCES


**Aguirre-Obando, O. A., A. J. Martins, and M. A. Navarro-Silva**. **2017**. First report of the Phe1534Cys *kdr* mutation in natural populations of *Aedes albopictus* from Brazil. Parasites and Vectors. 10: 1–10.

**Amraoui, F., W. Ben Ayed, Y. Madec, C. Faraj, O. Himmi, A. Btissam, M. Sarih, and A.-B. Failllouxid**. **2019**. Potential of *Aedes albopictus* to cause the emergence of arboviruses in Morocco. PLoS Negl. Trop. Dis. 13.

**Aponte, A. H., P. R. Penilla, F. Dzul-Manzanilla, A. Che-Mendoza, A. D. López, F. Solis, P. Manrique-Saide, H. Ranson, A. Lenhart, P. J. McCall, and A. D. Rodríguez**. **2013**. The pyrethroid resistance status and mechanisms in *Aedes aegypti* from the Guerrero state, Mexico. Pestic. Biochem. Physiol. 107: 226–234.

**Arslan, A., H. R. Rathor, M. U. Mukhtar, S. Mushtaq, A. Bhatti, M. Asif, I. Arshad, and J. F. Ahmad**. **2016**. Spatial distribution and insecticide susceptibility status of *Aedes aegypti* and *Aedes albopictus* in dengue affected urban areas of Rawalpindi, Pakistan. J. Vector Borne Dis. 53: 136–143.

**Balabanidou, V., L. Grigoraki, and J. Vontas**. **2018**. Insect cuticle: a critical determinant of insecticide resistance. Curr. Opin. Insect Sci. 27: 68–74.

**Bara, J., Z. Rapti, C. E. Cáceres, and E. J. Muturi**. **2015**. Effect of larval competition on extrinsic incubation period and vectorial capacity of *Aedes albopictus* for dengue virus. PLoS One. 10: 1–18.

**Barrett, A. D. T. 2018**. The reemergence of yellow fever. Science (80-. ). 361: 847–848.

**Bat-Miriam, M., and G. B. J. Craig**. **1966**. Mutants in *Aedes albopictus* (Diptera: Culicidae).

Mosq. News. 26: 13–22.

**Benedict, M. Q., R. S. Levine, W. A. Hawley, and L. P. Lounibos**. **2007**. Spread of the tiger: global risk of invasion by the mosquito *Aedes albopictus*. Vector-Borne Zoonotic Dis. 7: 76–85.

**Benelli, G., C. L. Jeffries, and T. Walker**. **2016**. Biological control of mosquito vectors: Past, present, and future. Insects. 7: 1–18.

**Bengoa, M., R. Eritja, S. Delacour, M. Á. Miranda, A. Sureda, and J. Lucientes**. **2017**. First Data on Resistance to Pyrethroids in Wild Populations of *Aedes albopictus* from Spain. J. Am. Mosq. Control Assoc. 33: 246–249.

**Bharati, M., and D. Saha**. **2017**. Insecticide susceptibility status and major detoxifying enzymes' activity in *Aedes albopictus* (Skuse), vector of dengue and chikungunya in Northern part of West Bengal, India. Acta Trop. 170: 112–119.

**Black, W. C. I., and N. M. DuTeau**. **1997**. RAPD-PCR and SSCP analysis for insect population genetic studies, pp. 361–373. *In* Crampton, J., Beard, C.B., Louis, C. (eds.), Mol. Biol. Insect Dis. Vectors. Chapman and Hall, New York.

**Bloomquist, J. R. 1996**. Ion Channels as Targets for Insecticides. Annu. Rev. Entomol. 41: 163–190.

**Bonds, J. A. S. 2012**. Ultra-low-volume space sprays in mosquito control: A critical review. Med. Vet. Entomol. 26: 121–130.

**Brengues, C., N. J. Hawkes, F. Chandre, L. McCarroll, S. Duchon, P. Guillet, S. Manguin, J. C. Morgan, and J. Hemingway**. **2003**. Pyrethroid and DDT cross-resistance in *Aedes aegypti* is correlated with novel mutations in the voltage-gated sodium channel gene. Med. Vet. Entomol. 17: 87–94.

**Brogdon, W. G., and J. C. McAllister**. **1998**. Insecticide resistance and vector control. Emerg. Infect. Dis. 4: 605–613.

**Brown, J. E., B. R. Evans, W. Zheng, V. Obas, L. Barrera-Martinez, A. Egizi, H. Zhao, A. Caccone, and J. R. Powell**. **2014**. Human impacts have shaped historical and recent evolution in *Aedes aegypti*, the dengue and yellow fever mosquito. Evolution (N. Y). 68: 514–525.

**Casas-Martinez, M., and J. L. Torres-Estrada**. **2003**. First Evidence of *Aedes albopictus* (Skuse) in Southern Chiapas, Mexico. Emerg. Infect. Dis. 9: 606–607.

**Casida, J. E., and R. J. Bryant**. **2017**. The ABCs of pesticide toxicology: Amounts, biology, and chemistry. Toxicol. Res. (Camb). 6: 755–763.

**Catterall, W. A. 2000**. From ionic currents to molecular mechanisms: The structure and function of voltage-gated sodium channels. Neuron. 26: 13–25.

**CDC**. **1989**. Update: *Aedes albopictus* Infestation – United States, Mexico. Morb. Mortal. Wkly. Rep. 38: 440, 445–446.

**CDC**. **2013**. CDC Bottle Bioassay. (https://www.cdc.gov/parasites/education_training/lab/bottlebioassay.html).

**CDC**. **2019a**. Dengue | Symptoms and Treatment. (https://www.cdc.gov/dengue/symptoms/index.html).

**CDC**. **2019b**. Information on aerial spraying. (https://www.cdc.gov/dengue/mosquito-control/aerial-spraying.html).

**Chambers, G. M., and M. J. Klowden**. **1994**. Nutritional reserves of autogenous and anautogenous selected strains of *Aedes albopictus* (Diptera: Culicidae). J. Med. Entomol. 31: 554–560.

**Chan, H. H., F. F. W. Mustafa, and J. Zairi**. **2011**. Assessing the susceptibility status of *Aedes albopictus* on Penang Island using two different assays. Trop. Biomed. 28: 464–470.

**Chan, H. H., and J. Zairi**. **2013**. Permethrin resistance in *Aedes albopictus* (Diptera: Culicidae) and associated fitness costs. J. Med. Entomol. 50: 362–370.

**Chan, Y. C., B. C. Ho, and K. L. Chan**. **1971**. *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse) in Singapore City. Bull. World Health Organ. 44: 651–658.

**Chang, C., W. K. Shen, T. T. Wang, Y. H. Lin, E. L. Hsu, and S. M. Dai**. **2009**. A novel amino acid substitution in a voltage-gated sodium channel is associated with knockdown resistance to permethrin in *Aedes aegypti*. Insect Biochem. Mol. Biol. 39: 272–278.

**Chareonviriyaphap, T. 2012**. Behavioral responses of mosquitoes to insecticides. Insectic. Eng. 201–220.

**Charlesworth, D., and J. H. Willis**. **2009**. The genetics of inbreeding depression. Nat. Rev. Genet. 10: 783–796.

**Charrel, R. N., X. de Lamballerie, and D. Raoult**. **2007**. Chikungunya outbreaks - The globalization of vectorborne diseases. N. Engl. J. Med. 356: 769–771.

**Chatterjee, M., S. Ballav, A. K. Maji, N. Basu, B. C. Sarkar, and P. Saha**. **2018**. Polymorphisms in voltage-gated sodium channel gene and susceptibility of *Aedes albopictus* to insecticides in three districts of northern West Bengal, India. PLoS Negl. Trop. Dis. 12: 1–15.

**Chen, H., K. Li, X. Wang, X. Yang, Y. Lin, F. Cai, W. Zhong, C. Lin, Z. Lin, and Y. Ma**. **2016**. First identification of *kdr* allele F1534S in VGSC gene and its association with resistance to pyrethroid insecticides in *Aedes albopictus* populations from Haikou City, Hainan Island, China. Infect. Dis. Poverty. 5: 1–8.

**Chuaycharoensuk, T., W. Juntarajumnong, W. Boonyuan, M. J. Bangs, P. Akratanakul, S. Thammapalo, N. Jirakanjanakit, S. Tanasinchayakul, and T. Chareonviriyaphap**. **2011**. Frequency of pyrethroid resistance in *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in Thailand. J. Vector Ecol. 36: 204–212.

**Crans, W. J., M. S. Chomsky, D. Guthrie, and A. Acquaviva**. **1996**. First record of *Aedes albopictus* from New Jersey. J. Am. Mosq. Control Assoc. 12: 307–309.

**Cui, K. L. 1982**. The autogeny of *Aedes albopictus* in Guangzhou area. Acta Entomol. Sin. 25: 256–259.

**Delatte, H., A. Desvars, A. Bouetard, S. Bord, G. Gimonneau, G. Vourc'h, and D. Fontenille**. **2010**. Blood-feeding behavior of *Aedes albopictus*, a vector of chikungunya on La Reunion. Vector-Borne Zoonotic Dis. 10: 249–258.

**Diniz, D. F. A., C. M. R. de Albuquerque, L. O. Oliva, M. A. V. de Melo-Santos, and C. F. J. Ayres**. **2017**. Diapause and quiescence: Dormancy mechanisms that contribute to the geographical expansion of mosquitoes and their evolutionary success. Parasites and Vectors. 10: 1–13.

**Du, Y., Y. Nomura, G. Satar, Z. Hu, R. Nauen, S. Y. He, B. S. Zhorov, and K. Dong**. **2013**. Molecular evidence for dual pyrethroid-receptor sites on a mosquito sodium channel. Proc. Natl. Acad. Sci. U. S. A. 110: 11785–11790.

**Eads, R. B. 1972**. Recovery of *Aedes albopictus* from used tires shipped to United States ports. Mosq. News. 32: 13–14.

**Effler, P. V, L. Pang, P. Kitsutani, V. Vorndam, M. Nakata, T. Ayers, J. Elm, T. Tom, P. Reiter, J. G. Rigau-Perez, J. M. Hayes, K. Mills, M. Napier, G. G. Clark, and D. J. Gubler**. **2005**. Dengue Fever, Hawaii, 2001-2002. Emerg. Infect. Dis. 11: 742–749.

**Enayati, A. A., H. Ranson, and J. Hemingway**. **2005**. Insect glutathione transferases and insecticide resistance. Insect Mol. Biol. 14: 3–8.

**Estep, A. S., N. D. Sanscrainte, C. M. Waits, S. J. Bernard, A. M. Lloyd, K. J. Lucas, E. A. Buckner, R. Vaidyanathan, R. Morreale, L. A. Conti, and J. J. Becnel**. **2018**. Quantification of permethrin resistance and *kdr* alleles in Florida strains of *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse). PLoS Negl. Trop. Dis. 12.

**Estrada-Franco, J. G., and G. B. J. Craig**. **1995**. Biology, Disease Relationships, and Control of *Aedes albopictus*. Pan Am. Heal. Organ. 42: 1–49.

**Faraji, A., and I. Unlu**. **2016**. The eye of the tiger, the thrill of the fight: Effective larval and adult control measures against the Asian tiger mosquito, *Aedes albopictus* (Diptera: Culicidae), in North America. J. Med. Entomol. 53: 1029–1047.

**Farnham, A. W. 1971**. Changes in cross-resistance patterns of houseflies selected with natural pyrethrins or resmethrin (5-benzyl-3-furylmethyl (±)-cis-trans-chrysanthemate). Pestic. Sci. 2: 138–143.

**Finlay, C. J. 1903**. New aspects of yellow fever etiology arising from experimental findings of the last three years. Public Health Pap. Rep. 29: 68–70.

**Flisser, A., A. Velasco-Villa, C. Martínez-Campos, F. González-Domínguez, B. Briseño-García, R. García-Suárez, A. Caballero-Servín, I. Hernández-Monroy, H. García-Lozano, L. Gutiérrez-Cogco, G. Rodríguez-Angeles, I. López-Martínez, S. Galindo-Virgen, R. Vázquez-Campuzano, S. Balandrano-Campos, C. Guzmán-Bracho, A. Olivo-Díaz, J. Luis de la Rosa, C. Magos, A. Escobar-Gutiérrez, and D. Correa**. **2002**. Infectious diseases in Mexico. A survey from 1995 - 2000. J. Clin. Microbiol. 33: 343–350.

**Floore, T. G. 2006**. Mosquito larval control practices: Past and present. J. Am. Mosq. Control

Assoc. 22: 527–533.

**Flores, A. E., G. Ponce, B. G. Silva, S. M. Gutierrez, C. Bobadilla, B. Lopez, R. Mercado, and W. C. Black IV**. **2013**. Wide spread cross resistance to pyrethroids in *Aedes aegypti* (Diptera: Culicidae) from Veracruz State Mexico. J. Econ. Entomol. 106: 959–69.

**Flores, A. E., G. Reyes Solis, I. F. Salas, F. J. Sanchez Ramos, and G. Ponce Garcia**. **2009**. Resistance to permethrin in *Aedes aegypti* (L.) in Northern Mexico. Southwest. Entomol. 34: 167–177.

**Flores, H. A., and S. L. O'Neill**. **2018**. Controlling vector-borne diseases by releasing modified mosquitoes. Nat. Rev. Microbiol. 16: 508–518.

**Forattini, O. P. 1986**. Identification of *Aedes* (*Stegomyia*) *albopictus* (Skuse) in Brazil. Rev. Saude Publica. 20: 244–245.

**Foster, W. A., and E. D. Walker**. **2002**. Mosquitoes (Culicidae), pp. 203–262. *In* Med. Vet. Entomol.

**Gantz, V. M., N. Jasinskiene, O. Tatarenkova, A. Fazekas, V. M. Macias, E. Bier, and A. A. James**. **2015**. Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*. Proc. Natl. Acad. Sci. U. S. A. 112: E6736–E6743.

**Gao, J.-P., H.-M. Chen, H. Shi, H. Peng, and Y.-J. Ma**. **2018**. Correlation between adult pyrethroid resistance and knockdown resistance (*kdr*) mutations in *Aedes albopictus* (Diptera: Culicidae) field populations in China. Infect. Dis. Poverty. 7: 1–9.

**Garcia-Luna, S. M., J. Weger-Lucarelli, C. Rückert, R. A. Murrieta, M. C. Young, A. D. Byas, J. R. Fauver, R. Perera, A. E. Flores-Suarez, G. Ponce-Garcia, A. D. Rodriguez, G. D. Ebel, and W. C. Black**. **2018**. Variation in competence for ZIKV transmission by

*Aedes aegypti* and *Aedes albopictus* in Mexico. PLoS Negl. Trop. Dis. 12: 1–21.

**García, G. P., A. E. Flores, I. Fernández-Salas, K. Saavedra-Rodríguez, G. Reyes-Solis, S. Lozano-Fuentes, J. G. Bond, M. Casas-Martínez, J. M. Ramsey, J. García-Rejón, M. Domínguez-Galera, H. Ranson, J. Hemingway, L. Eisen, and W. C. Black IV**. **2009**. Recent rapid rise of a permethrin knock down resistance allele in *Aedes aegypti* in México. PLoS Negl. Trop. Dis. 3.

**Gatt, P., J. Deeming, and F. Schaffner**. **2009**. First record of *Aedes* (*Stegomyia*) *albopictus* (Skuse) (Diptera: Culicidae) in Malta. Eur. Mosq. Bull. 27: 56–64.

**Germer, S., and R. Higuchi**. **1999**. Single-tube genotyping without oligonucleotide probes. Genome Res. 9: 72–78.

**Gilbertson, W. E. 1945**. Sanitary aspects of the control of the 1943-1944 epidemic of dengue fever in Honolulu. Am. J. Public Heal. Nations Heal. 35: 261–270.

**Gorgas, W. C. 1915**. Sanitation in Panama. Appleton.

**Gratz, N. G. 2004**. Critical review of the vector status of *Aedes albopictus*. Med. Vet. Entomol. 18: 215–227.

**Gray, L., S. D. Florez, A. M. Barreiro, J. Vadillo-Sánchez, G. González-Olvera, A. Lenhart, P. Manrique-Saide, and G. M. Vazquez-Prokopec**. **2018**. Experimental evaluation of the impact of household aerosolized insecticides on pyrethroid resistant *Aedes aegypti*. Sci. Rep. 8: 1–11.

**Gubler, D. J. 1998**. Dengue and dengue hemorrhagic fever. Clin. Microbiol. Rev. 11: 480–496.

**Haddi, K., H. V. V. Tomé, Y. Du, W. R. Valbon, Y. Nomura, G. F. Martins, K. Dong, and E. E. Oliveira**. **2017**. Detection of a new pyrethroid resistance mutation (V410L) in the sodium channel of *Aedes aegypti*: A potential challenge for mosquito control. Sci. Rep. 7:

1–9.

**Hahn, M. B., L. Eisen, J. McAllister, H. M. Savage, J. P. Mutebi, and R. J. Eisen**. **2017**. Updated reported distribution of *Aedes* (*Stegomyia*) *aegypti* and *Aedes* (*Stegomyia*) *albopictus* (Diptera: Culicidae) in the United States, 1995–2016. J. Med. Entomol. 54: 1420–1424.

**Hammond, A., R. Galizi, K. Kyrou, A. Simoni, C. Siniscalchi, D. Katsanos, M. Gribble, D. Baker, E. Marois, S. Russell, A. Burt, N. Windbichler, A. Crisanti, and T. Nolan**. **2016**. A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae.* Nat. Biotechnol. 34: 78–83.

**Hamzah, S. N., and Z. Alias**. **2016**. Purification, expression and partial characterization of glutathione *S*-transferases (GSTs) from three different strains of *Aedes albopictus* (Diptera: Culicidae). Trop. Biomed. 33: 335–347.

**Harris, A. F., S. Rajatileka, and H. Ranson**. **2010**. Pyrethroid resistance in *Aedes aegypti* from Grand Cayman. Am. J. Trop. Med. Hyg. 83: 277–284.

**Hawley, W. A. 1988**. The biology of *Aedes albopictus*. J.Amer.Mosquito.Contr.Assn. 4: 1–39.

**Hawley, W. A., P. Reiter, R. S. Copeland, C. B. Pumpuni, and G. B. Craig**. **1987**. *Aedes albopictus* in North America : Probable introduction in used tires from Northern Asia. Science (80-. ). 236: 1114–1116.

**Heinisch, M. R. S., F. A. Diaz-Quijano, F. Chiaravalloti-Neto, F. G. M. Pancetti, R. R. Coelho, P. dos Santos Andrade, P. R. Urbinatti, R. M. M. Sá De Almeida, and T. N. Lima-Camara**. **2019**. Seasonal and spatial distribution of *Aedes aegypti* and *Aedes albopictus* in a municipal urban park in São Paulo, SP, Brazil. Acta Trop. 189: 104–113.

**Hemingway, J. 1998**. Techniques to detect insecticide resistance mechanisms (Field and

laboratory manual). World Heal. Organ. WHO/CDC/CP.

**Hemingway, J., N. J. Hawkes, L. McCarroll, and H. Ranson**. **2004**. The molecular basis of insecticide resistance in mosquitoes. Insect Biochem. Mol. Biol. 34: 653–665.

**Hien, D. S. 1976**. Biology of *Aedes Aegypti* (L., 1762) and *Aedes albopictus* (Skuse, 1895) (Diptera: Culicidae).IV. The feeding of females. Acta Parasitol. Pol. 24: 27–35.

**Hofhuis, A., J. Reimerink, C. Reusken, E.-J. Scholte, A. de Boer, W. Takken, and M. Koopmans**. **2009**. The hidden passenger of lucky bamboo: Do imported *Aedes albopictus* mosquitoes cause dengue virus transmission in the Netherlands? Vector-Borne Zoonotic Dis. 9: 217–220.

**Housset, P., and R. Dickmann**. **2009**. A promise fulfilled – pyrethroid development and the benefits for agriculture and human health. Bayer Crop. J. 62: 135–144.

**Howard, L. O. 1910**. Preventative and remedial work against mosquitoes. U.S. Dep. Agric. Bur. Entomol. Bulletin N: 1–126.

**Ibáñez-Bernal, Sergio; Martínez-Campos, C. 1994**. *Aedes albopictus* in Mexico. J. Am. Mosq. Control Assoc. 10: 231–232.

**Ishak, I. H., Z. Jaal, H. Ranson, and C. S. Wondji**. **2015**. Contrasting patterns of insecticide resistance and knockdown resistance (*kdr*) in the dengue vectors *Aedes aegypti* and *Aedes albopictus* from Malaysia. Parasites and Vectors. 8: 1–13.

**Ishak, I. H., J. M. Riveron, S. S. Ibrahim, R. Stott, J. Longbottom, H. Irving, and C. S. Wondji**. **2016**. The Cytochrome P450 gene CYP6P12 confers pyrethroid resistance in *kdr*-free Malaysian populations of the dengue vector *Aedes albopictus*. Sci. Rep. 6: 1–13.

**Joyce, C. R. 1961**. Potentialities for accidental establishment of exotic mosquitoes in Hawaii. Proceedings, Hawaiian Entomol. Soc. 17: 403–413.

**Kambhampati, S., W. C. Black, and K. S. Rai**. **1991**. Geographic origin of the US and Brazilian *Aedes albopictus*. Heredity (Edinb). 67: 85–94.

**Kamgang, B., S. Marcombe, F. Chandre, E. Nchoutpouen, P. Nwane, J. Etang, V. Corbel, and C. Paupy**. **2011**. Insecticide susceptibility of *Aedes aegypti* and *Aedes albopictus* in Central Africa. Parasites and Vectors. 4: 1–8.

**Kamgang, B., E. Nchoutpouen, F. Simard, and C. Paupy**. **2012**. Notes on the blood-feeding behavior of *Aedes albopictus* (Diptera: Culicidae) in Cameroon. Parasites and Vectors. 5.

**Kamgang, B., A. P. Yougang, M. Tchoupo, J. M. Riveron, and C. Wondji**. **2017**. Temporal distribution and insecticide resistance profile of two major arbovirus vectors *Aedes aegypti* and *Aedes albopictus* in Yaoundé, the capital city of Cameroon. Parasit. Vectors. 10: 469.

**Karunaratne, S. H. P. P., T. C. Weeraratne, M. D. B. Perera, and S. N. Surendran**. **2013**. Insecticide resistance and efficacy of space spraying and larviciding in the control of dengue vectors *Aedes aegypti* and *Aedes albopictus* in Sri Lanka. Pestic. Biochem. Physiol. 107: 98–105.

**Kasai, S., B. Caputo, T. Tsunoda, T. C. Cuong, Y. Maekaw, S. G. Lam-Phua, V. Pichler, K. Itokawa, K. Murota, O. Komagata, C. Yoshida, H. H. Chung, R. Bellini, Y. Tsuda, H. J. Teng, J. L. de Lima Filho, L. C. Alves, L. C. Ng, N. Minakawa, N. T. Yen, T. V. Phong, K. Sawabe, and T. Tomita**. **2019**. First detection of a Vssc allele V1016G conferring a high level of insecticide resistance in *Aedes albopictus* collected from Europe (Italy) and Asia (Vietnam), 2016: A new emerging threat to controlling arboviral diseases. Eurosurveillance. 24: 1–12.

**Kasai, S., L. C. Ng, S. G. Lam-Phua, C. S. Tang, K. Itokawa, O. Komagata, M. Kobayashi, and T. Tomita**. **2011**. First detection of a putative knockdown resistance gene in major

mosquito vector, *Aedes albopictus*. Jpn. J. Infect. Dis. 64: 217–221.

**Kauffman, E. B., and L. D. Kramer**. **2017**. Zika virus mosquito vectors: Competence, biology, and vector control. J. Infect. Dis. 216: S976–S990.

**Kramer, L. D., and A. T. Ciota**. **2015**. Dissecting vectorial capacity for mosquito-borne viruses. Curr. Opin. Virol. 15: 112–118.

**Kuri-Morales, P. A., F. Correa-Morales, C. Gonzalez-Acosta, M. Moreno-Garcia, R. Santos-Luna, S. Roman-Perez, F. Salazar-Penagos, M. Lombera-Gonzalez, G. Sanchez-Tejeda, and J. F. Gonzalez-Roldan**. **2018**. Insecticide susceptibility status in Mexican populations of *Stegomyia aegypti* ( = *Aedes aegypti* ): a nationwide assessment. Med. Vet. Entomol. 32: 162–174.

**Kushwah, R. B. S., C. L. Dykes, N. Kapoor, T. Adak, and O. P. Singh**. **2015**. Pyrethroid-resistance and presence of two knockdown resistance (*kdr*) mutations, F1534C and a novel mutation T1520I, in Indian *Aedes aegypti*. PLoS Negl. Trop. Dis. 9.

**Kushwah, R. B. S., P. K. Mallick, H. Ravikumar, V. Dev, N. Kapoor, T. Adak, and O. P. Singh**. **2015**. Status of DDT and pyrethroid resistance in Indian *Aedes albopictus* and absence of knockdown resistance (*kdr*) mutation. J. Vector Borne Dis. 52: 95–98.

**Lee, B. 1905**. A tribute to Carlos J. Finlay for his distinguished services to science and humanity in the discovery of the mode of propagation of yellow fever. Public Health Pap. Rep. 30: 8–14.

**Lei, H. Y., T. M. Yeh, H. S. Liu, Y. S. Lin, S. H. Chen, and C. C. Liu**. **2001**. Immunopathogenesis of dengue virus infection. J. Biomed. Sci. 8: 377–388.

**Li, Y., J. Xu, D. Zhong, H. Zhang, W. Yang, G. Zhou, X. Su, Y. Wu, K. Wu, S. Cai, G. Yan, and X.-G. Chen**. **2018**. Evidence for multiple-insecticide resistance in urban *Aedes*

*albopictus* populations in southern China. Parasites and Vectors. 11: 1–10.

**Liu, H., E. W. Cupp, A. Guo, and N. Liu**. **2004**. Insecticide resistance in Alabama and Florida mosquito strains of *Aedes albopictus*. J. Med. Entomol. 41: 946–952.

**Liu, N. 2015**. Insecticide resistance in mosquitoes: Impact, mechanisms, and research directions. Annu. Rev. Entomol. 60: 537–559.

**Lopez-Monroy, B., S. M. Gutierrez-Rodriguez, O. K. Villanueva-Segura, G. Ponce-Garcia, F. Morales-Forcada, L. C. Alvarez, and A. E. Flores**. **2018**. Frequency and intensity of pyrethroid resistance through the CDC bottle bioassay and their association with the frequency of *kdr* mutations in *Aedes aegypti* (Diptera: Culicidae) from Mexico. Pest Manag. Sci. 74: 2176–2184.

**Loroño-Pino, M. A., Y. N. Chan-Dzul, R. Zapata-Gil, C. Carrillo-Solís, A. Uitz-Mena, J. E. García-Rejón, T. J. Keefe, B. J. Beaty, and L. Eisen**. **2014**. Household use of insecticide consumer products in a dengue-endemic area in México. Trop. Med. Int. Heal. 19: 1267–1275.

**Marcombe, S., R. Blanc Mathieu, N. Pocquet, M.-A. Riaz, R. Poupardin, S. Sélior, F. Darriet, S. Reynaud, A. Yébakima, V. Corbel, J.-P. David, and F. Chandre**. **2012**. Insecticide resistance in the dengue vector *Aedes aegypti* from Martinique: Distribution, mechanisms and relations with environmental factors. PLoS One. 7.

**Marcombe, S., A. Farajollahi, S. P. Healy, G. G. Clark, and D. M. Fonseca**. **2014**. Insecticide resistance status of United States populations of *Aedes albopictus* and mechanisms involved. PLoS One. 9: e101992.

**McAllister, J. C., M. S. Godsey, and M. L. Scott**. **2012**. Pyrethroid resistance in *Aedes aegypti* and *Aedes albopictus* from Port-au-Prince, Haiti. J. Vector Ecol. 37: 325–332.

**Metselaar, D., C. R. Grainger, K. G. Oei, D. G. Reynolds, M. Pudney, C. J. Leake, P. M. Tukei, R. M. D'Offay, and D. I. H. Simpson**. **1980**. An outbreak of type 2 dengue fever in the Seychelles, probably transmitted by *Aedes albopictus* (Skuse). Bull. World Health Organ. 58: 937–943.

**Mogi, M., and N. Tuno**. **2014**. Impact of climate change on the distribution of *Aedes albopictus* (Diptera: Culicidae) in Northern Japan: Retrospective analyses. J. Med. Entomol. 51: 572–579.

**Monath, T. P. 1988**. The Arboviruses: epidemiology and ecology. CRC Press, Boca Raton, FL.

**Monath, T. P. 2001**. Yellow fever: An update. Lancet Infect. Dis. 1: 11–20.

**Montella, I. R., R. Schama, and D. Valle**. **2012**. The classification of esterases: An important gene family involved in insecticide resistance - A review. Mem. Inst. Oswaldo Cruz. 107: 437–449.

**Moore, C. G., D. B. Francy, D. A. Eliason, and T. P. Monath**. **1988**. *Aedes albopictus* in the United States: Rapid spread of a potential disease vector. J. Am. Mosq. Control Assoc. 4: 356–361.

**Mori, A., J. Romero-Severson, W. C. Black IV, and D. W. Severson**. **2008**. Quantitative trait loci determining autogeny and body size in the Asian tiger mosquito (*Aedes albopictus*). Heredity (Edinb). 101: 75–82.

**Namadurai, S., N. R. Yereddi, F. S. Cusdin, C. L.-H. Huang, D. Y. Chirgadze, and A. P. Jackson**. **2015**. A new look at sodium channel β subunits. Open Biol. 5.

**Niebylski, M. L., and G. B. J. Craig**. **1994**. Dispersal and survival of *Aedes albopictus* at a scrap tire yard in Missouri. J. Am. Mosq. Control Assoc. 10: 339–343.

**Niebylski, M. L., H. M. Savage, R. S. Nasci, and G. B. J. Craig**. **1994**. Blood hosts of *Aedes*

*albopictus* in the United States. J. Am. Mosq. Control Assoc. 10: 447–450.

**Ogata, K., and A. L. Samayoa**. **1996**. Discovery of *Aedes albopictus* in Guatamala. J. Am. Mosq. Control Assoc. 12: 503–506.

**Okimoto, R., and J. B. Dodgson**. **1996**. Improved PCR amplification of multiple specific alleles (PAMSA) using internally mismatched primers. Biotechniques. 21: 20–26.

**Orta-Pesina, H., R. Mercado-Hernandez, and M. A. Valdez-Rodriquez**. **2001**. *Aedes albopictus* in Allende City, Nuevo León, Mexico. J. Am. Mosq. Control Assoc. 17: 260–261.

**Ortega-morales, A. I., P. Mis-Avila, M. Dominguez-Galera, G. Canul-Amaro, J. Esparza-Aguilar, J. Carlos-Azueta, S. Badillo-Perry, P. Marin, J. Polanco, and I. Fernandez-Salas**. **2010**. First Record of *Stegomyia albopicta* (*Aedes albopictus*) in Belize. Southwest. Entomol. 35: 197–198.

**Ortega-Morales, A. I., and Q. K. S. Rodríguez**. **2016**. First record of *Aedes albopictus* (Diptera: Culicidae) in San Luis Potosi, Mexico. J. Vector Ecol. 41: 314–315.

**Paupy, C., H. Delatte, L. Bagny, V. Corbel, and D. Fontenille**. **2009**. *Aedes albopictus*, an arbovirus vector: From the darkness to the light. Microbes Infect. 11: 1177–1185.

**Pech-May, A., D. A. Moo-Llanes, M. Belem Puerto-Avila, M. Casas, R. Danis-Lozano, G. Ponce, E. Tun-Ku, J. Francisco Pinto-Castillo, A. Villegas, C. R. Ibáñez-Piñon, C. González, and J. M. Ramsey**. **2016**. Population genetics and ecological niche of invasive *Aedes albopictus* in Mexico. Acta Trop. 157: 30–41.

**Pereira da-Cunha, M., J. Bento Pereira Lima, W. G. Brogdon, G. Efrain Moya, and D. Valle**. **2005**. Monitoring of resistance to the pyrethroid cypermethrin in Brazilian *Aedes aegypti* (Diptera: Culicidae) populations collected between 2001 and 2003. Mem. Inst.

Oswaldo Cruz. 100: 441–444.

**Petersen, L. R., D. J. Jamieson, A. M. Powers, and M. A. Honein**. **2016**. Zika virus. N. Engl. J. Med. 374: 1552–1563.

**Pichler, V., R. Bellini, R. Veronesi, D. Arnoldi, A. Rizzoli, R. P. Lia, D. Otranto, F. Montarsi, S. Carlin, M. Ballardini, E. Antognini, M. Salvemini, E. Brianti, G. Gaglio, M. Manica, P. Cobre, P. Serini, E. Velo, J. Vontas, I. Kioulos, J. Pinto, A. della Torre, and B. Caputo**. **2018**. First evidence of resistance to pyrethroid insecticides in Italian *Aedes albopictus* populations 26 years after invasion. Pest Manag. Sci. 74: 1319–1327.

**Ponlawat, A., J. G. Scott, and L. C. Harrington**. **2005**. Insecticide Susceptibility of *Aedes aegypti* and *Aedes albopictus* across Thailand. J. Med. Entomol. 42: 821–825.

**Pratt, J. J. J., R. H. Heterick, J. B. Harrison, and L. Haber**. **1946**. Tires as a factor in the transportation of mosquitoes by ships. Mil. Surg. 99: 785–788.

**Priester, T. M., and G. P. Georghiou**. **1978**. Induction of high resistance to permethrin in *Culex pipiens quinquefasciatus*. J. Econ. Entomol. 71: 197–200.

**Ranson, H., J. Burhani, N. Lumjuan, and W. C. Black IV**. **2010**. Insecticide resistance in dengue vectors. Trop. [online]. 1: 1–12.

**Reiter, P. 1998**. *Aedes albopictus* and the world trade in used tires, 1988-1995: The shape of things to come? Joumal Am. Mosq. Control Assoc.

**Reiter, P., and R. F. J. Darsie**. **1984**. *Aedes albopictus* in Memphis, Tennessee (USA): An achievement of modern transportation? Mosq. News. 44: 396–399.

**Reiter, P., D. Fontenille, and C. Paupy**. **2006**. *Aedes albopictus* as an epidemic vector of chikungunya virus: another emerging problem? Lancet Infect. Dis. 6: 463–464.

**Richards, S. L., J. A. G. Balanay, A. V. White, J. Hope, K. Vandock, B. D. Byrd, and M. H.**

**Reiskind**. **2017**. Insecticide susceptibility screening against *Culex* and *Aedes* (Diptera: Culicidae) mosquitoes from the United States. J. Med. Entomol. 55: 398–407.

**Richards, S. L., L. Ponnusamy, T. R. Unnasch, H. K. Hassan, and C. S. Apperson**. **2006**. Host-feeding patterns of *Aedes albopictus* (Diptera: Culicidae) in relation to availability of human and domestic animals in suburban landscapes of central North Carolina. J. Med. Entomol. 43: 543–551.

**Rodriguez Tovar, M. L., and M. G. Ortega Martinez**. **1994**. *Aedes albopictus* in Muzquiz City, Coahuila, Mexico. J. Am. Mosq. Control Assoc. 10: 587.

**Roizid, D., A. L. Wilson, T. W. Scott, D. M. Fonseca, F. Jourdain, Pi. Müller, R. Velayudhan, and V. Corbel**. **2018**. Integrated *Aedes* management for the control of *Aedes*-borne diseases. PLoS Negl. Trop. Dis. 12: 1–21.

**Ross, R. W. 1956**. The Newala epidemic: III. The virus: Isolation, pathogenic properties and relationship to the epidemic. J. Hyg. (Lond). 54: 177–191.

**Saavedra-Rodriguez, K., F. V. Maloof, C. L. Campbell, J. Garcia-Rejon, A. Lenhart, P. Penilla, A. Rodriguez, A. A. Sandoval, A. E. Flores, G. Ponce, S. Lozano, and W. C. Black**. **2018**. Parallel evolution of vgsc mutations at domains IS6, IIS6 and IIIS6 in pyrethroid resistant *Aedes aegypti* from Mexico. Sci. Rep. 8: 1–9.

**Saavedra-Rodriguez, K., A. F. Suarez, I. F. Salas, C. Strode, H. Ranson, J. Hemingway, and W. C. Black IV**. **2012**. Transcription of detoxification genes after permethrin selection in the mosquito *Aedes aegypti*. Insect Mol. Biol. 21: 61–77.

**Saavedra-Rodriguez, K., L. Urdaneta-Marquez, S. Rajatileka, A. E. Flores, I. Fernandez-Salas, J. Bisset, M. Rodriguez, P. J. Mccall, M. J. Donnelly, H. Ranson, J. Hemingway, and W. C. Black IV**. **2007**. A mutation in the voltage-gated sodium channel gene

associated with pyrethroid resistance in Latin American *Aedes aegypti*. 16: 785–798.

**Sabin, A. B. 1952**. Research on dengue during World War II. Am. J. Trop. Med. Hyg. 30–50.

**Salomón-Grajales, J., G. V. Lugo-Moguel, V. R. Tinal-Gordillo, J. de La Cruz-Velázquez, B. J. Beaty, L. Eisen, S. Lozano-Fuentes, C. G. Moore, and J. E. García-Rejón**. **2012**. *Aedes albopictus* mosquitoes, Yucatan Peninsula, Mexico. Emerg. Infect. Dis. 18: 525–527.

**Sames IV, W. J., R. Bueno Jr, J. Hayes, and J. K. Olson**. **1996**. Instecticide susceptibility of *Aedes aegypti* and *Aedes albopictus* in the lower Rio Grande Valley of Texas and Mexico. J. Am. Mosq. Control Assoc. 12: 487–490.

**Sanchez-Vargas, I., L. C. Harrington, W. C. Black IV, and K. E. Olson**. **2019**. Analysis of salivary glands and saliva from *Aedes albopictus* and *Aedes aegypti* infected with chikungunya viruses. Insects. 10.

**Savage, H. M., M. L. Niebylski, G. C. Smith, C. J. Mitchell, and G. B. J. Craig**. **1993**. Host-feeding patterns of *Aedes albopictus* (Diptera: Culicidae) at a temperate North American site. J. Med. Entomol. 30: 27–34.

**Schliessmann, D. J. 1964**. The *Aedes aegypti* eradication program of the U.S. Mosq. News. 24: 124–132.

**Schliessmann, D. J. 1967**. Initiation of the *Aedes aegypti* eradication programme of the USA, Bull. World Health Organ.

**Secretaría de Salud**. **2015**. Lista actualizada de productos recomendados por el CENAPRECE para el combate de insectos vectores de enfermedades a partir de 2015.

**Secretaría de Salud**. **2016**. Lista actualizada de productos recomendados por el CENAPRECE para el combate de insectos vectores de enfermedades a partir de 2016.

**Selvi, S., M. A. Edah, W. A. Nazni, H. L. Lee, B. K. Tyagi, M. Sofian-Azirun, and A. H.**

**Azahari**. **2010**. Insecticide susceptibility and resistance development in malathion selected *Aedes albopictus* (Skuse). Trop. Biomed. 27: 534–550.

**Shaw, W. R., and F. Catteruccia**. **2019**. Vector biology meets disease control: using basic research to fight vector-borne diseases. Nat. Microbiol. 4: 20–34.

**Simon, F., P. Parola, M. Grandadam, S. Fourcade, M. Oliver, P. Brouqui, P. Hance, P. Kraemer, A. A. Mohamed, X. de Lamballerie, R. Charrel, and H. Tolou**. **2007**. Chikungunya infection: An emerging rheumatism among travelers returned from Indian Ocean Islands. Report of 47 cases. Medicine (Baltimore). 86: 123–137.

**Slosek, J. 1986**. *Aedes aegypti* mosquitoes in the Americas: A review of their interactions with the human population. Soc. Sci. Med. 23: 249–257.

**Soderlund, D. M. 2010**. State-dependent modification of voltage-gated sodium channels by pyrethroids. Pestic. Biochem. Physiol. 97: 78–86.

**Soderlund, D. M., and J. R. Bloomquist**. **1989**. Neurotoxic actions of pyrethroid insecticides. Annu. Rev. Entomol. 34: 77–96.

**Sprenger, D., and T. Wuithiranyagool**. **1986**. The discovery and distribution of *Aedes albopictus* in Harris County, Texas. J.Amer.Mosquito.Contr.Assn. 2: 217–219.

**Srisawat, R., N. Komalamisra, Y. Eshita, M. Zheng, K. Ono, T. Q. Itoh, A. Matsumoto, S. Petmitr, and Y. Rongsriyam**. **2010**. Point mutations in domain II of the voltage-gated sodium channel gene in deltamethrin-resistant *Aedes aegypti* (Diptera: Culicidae). Appl. Entomol. Zool. 45: 275–282.

**Strode, C., C. S. Wondji, J. P. David, N. J. Hawkes, N. Lumjuan, D. R. Nelson, D. R. Drane, S. H. P. P. Karunaratne, J. Hemingway, W. C. Black IV, and H. Ranson**. **2008**. Genomic analysis of detoxification genes in the mosquito *Aedes aegypti*. Insect Biochem.

Mol. Biol. 38: 113–123.

**Tabachnick, W. J. 1991**. Evolutionary genetics and arthropod-borne disease: The yellow fever mosquito. Am. Entomol. 37: 14–26.

**Theiler, M., and H. H. Smith**. **1937**. The use of yellow fever virus modified by in vitro cultivation for human immunization. J. Exp. Med. 65: 787–800.

**Torres-Avendaño, J. I., H. Castillo-Ureta, E. H. Torres-Montoya, E. Meza-Carrillo, R. L. Lopez-Mendoza, M. G. Vazquez-Martinez, and J. G. Rendon-Maldonado**. **2015**. First record of *Aedes albopictus* in Sinaloa, Mexico. J. Am. Mosq. Control Assoc. 31: 164–166.

**Tsetsarkin, K. A., D. L. Vanlandingham, C. E. McGee, and S. Higgs**. **2007**. A single mutation in chikungunya virus affects vector specificity and epidemic potential. PLoS Pathog. 3: 1895–1906.

**Tsetsarkin, K. A., and S. C. Weaver**. **2011**. Sequential adaptive mutations enhance efficient vector switching by chikungunya virus and its epidemic emergence. PLoS Pathog. 7.

**Unlu, I., A. Farajollahi, D. Strickman, and D. M. Fonseca**. **2013**. Crouching tiger, hidden trouble: Urban sources of *Aedes albopictus* (Diptera: Culicidae) refractory to source-reduction. PLoS One. 8: 1–12.

**Vais, H., M. S. Williamson, A. L. Devonshire, and P. N. Usherwood**. **2001**. The molecular interactions of pyrethroid insecticides with insect and mammalian sodium channels. Pest Manag. Sci. 57: 877–888.

**Valença, M. A., L. S. Marteis, L. M. Steffler, A. M. Silva, and R. L. C. Santos**. **2013**. Dynamics and characterization of *Aedes aegypti* (L.) (Diptera: Culicidae) key breeding sites. Neotrop. Entomol. 42: 311–316.

**Valle, D., I. Montella, P. Medeiros, R. Ribeiro, and A. Martins**. **2006**. Quantification

methodology for enzyme activity related to insecticide resistance in *Aedes aegypti*. Brasília Ministério da Saúde/Brasil.

**Vannice, K. S., A. Drubin, and J. Hombach**. **2016**. Status of vaccine research and development of vaccines for dengue. Vaccine. 34: 2934–2938.

**Vazeille, M., L. Rosen, L. Mousson, and A.-B. Failloux**. **2003**. Low oral receptivity for dengue type 2 viruses of *Aedes albopictus* from Southeast Asia compared with that of *Aedes aegypti*. Am. J. Trop. Med. Hyg. 68: 203–208.

**Vera-Maloof, F. Z., K. Saavedra-Rodriguez, A. E. Elizondo-Quiroga, S. Lozano-Fuentes, and W. C. Black IV**. **2015**. Coevolution of the Ile1,016 and Cys1,534 mutations in the voltage gated sodium channel gene of *Aedes aegypti* in Mexico. PLoS Negl. Trop. Dis. 9: 1–22.

**Vijayakumar, K., T. K. Sudheesh Kumar, Z. T. Nujum, F. Umarul, and A. Kuriakose**. **2014**. A study on container breeding mosquitoes with special reference to *Aedes* (*Stegomyia*) *aegypti* and *Aedes albopictus* in Thiruvananthapuram district, India. J. Vector Borne Dis. 51: 27–32.

**Villegas-Trejo, A., P. Manrique-Saide, A. Che-Mendoza, W. Cruz-Canto, M. G. Fernández, C. González-Acosta, F. Dzul-Manzanilla, H. Huerta, and J. I. Arredondo-Jiménez**. **2010**. First report of *Aedes albopictus* and oother mosquito species in Morelos, Mexico. J. Am. Mosq. Control Assoc. 26: 321–323.

**Vontas, J., E. Kioulos, N. Pavlidi, E. Morou, A. della Torre, and H. Ranson**. **2012**. Insecticide resistance in the major dengue vectors *Aedes albopictus* and *Aedes aegypti*. Pestic. Biochem. Physiol. 104: 126–131.

**Wan-Norafikah, O., W. A. Nazni, H. L. Lee, P. Zainol-Ariffin, and M. Sofian-Azirun**. **2013**.

Susceptibility of *Aedes albopictus* Skuse (Diptera: Culicidae) to permethrin in Kuala Lumpur, Malaysia. Asian Biomed. 7: 51–62.

**Weaver, S. C., and W. K. Reisen**. **2010**. Present and future arboviral threats. Antiviral Res. 85: 328–345.

**WHO/CDC/WHOPES/GCDPP**. **2005**. Safety of pyrethroids for public health use. World Heal. Organ.

**WHO**. **2012**. Global plan for insecticide resistance management in malaria vectors.

**WHO**. **2017**. Situation report. Zika virus. Microcephaly. Guillain-Barre syndrome. 2 February 2017.

**WHO Expert Committee on Insecticides & World Health Organization**. **1986**. Resistance of vectors and reservoirs of disease to pesticides: tenth report of the WHO expert committee on vector biology and control [meeting held in Geneva from 5 to 11 November 1985].

**World Health Organization**. **2013**. Test procedures for insecticide resistance monitoring in malaria vector mosquitoes.

**Xu, J., M. Bonizzoni, D. Zhong, G. Zhou, S. Cai, Y. Li, X. Wang, E. Lo, R. Lee, R. Sheen, J. Duan, G. Yan, and X.-G. Chen**. **2016**. Multi-country survey revealed prevalent and novel F1534S mutation in voltage-gated sodium channel (VGSC) gene in *Aedes albopictus*. PLoS Negl. Trop. Dis. 10: 1–12.

**Yanola, J., P. Somboon, C. Walton, W. Nachaiwieng, P. Somwang, and L. Prapanthadara**. **2011**. High-throughput assays for detection of the F1534C mutation in the voltage-gated sodium channel gene in permethrin-resistant *Aedes aegypti* and the distribution of this mutation throughout Thailand. Trop. Med. Int. Heal. 16: 501–509.

**Yen, C., T. B. Hyde, A. J. Costa, K. Fernandez, J. S. Tam, S. Hugonnet, A. M. Huvos, P.**

**Duclos, V. J. Dietz, and B. T. Burkholder**. **2015**. The development of global vaccine stockpiles. Lancet Infect. Dis. 15: 340–347.

**Yu, S. J. 2015**. The toxicology and biochemistry of insecticides, 2nd ed. CRC Press.

**Zhou, X., C. Yang, N. Liu, M. Li, Y. Tong, X. Zeng, and X. Qiu**. **2019**. Knockdown resistance (*kdr*) mutations within seventeen field populations of *Aedes albopictus* from Beijing China: First report of a novel V1016G mutation and evolutionary origins of *kdr* haplotypes. Parasit. Vectors. 12.

## APPENDIX: SUPPLEMENTAL INFORMATION

### Additional Information on Mosquitoes Collected from Chiapas, Mexico

Members of our lab, along with other colleagues from the Microbiology, Immunology, and Pathology department at CSU, collected *Ae. albopictus* (along with *Ae. aegypti*) eggs from areas in and around the city of Tapachula, Chiapas, with the help of our collaborators at Centro Regional de Investigación en Salud Pública (CRISP) between August and September 2016. Within the city of Tapachula, larvae and pupae were collected from a wide variety of containers that were in and around people's yards (tires, cups, buckets, outdoor sinks, etc.). Mosquitoes from the rural towns outside of the city were primarily collected from flowerpots and vases within cemeteries. The collected larvae and pupae ($F_0$) were brought back to the insectary at CRISP and were identified and sorted by species upon emergence. Adults were blood fed and $F_1$ eggs were collected and sent to CSU.

### Additional Information on Mosquitoes Collected from Weslaco, Texas

Initially the Gabriel Hamer Lab provided us *Ae. albopictus* eggs from five sites in Weslaco, however two of the collections (Valley Nature Center and Weslaco Mile 5) did not produce enough eggs in the laboratory and could not be maintained. The three remaining colonies were used for this study. *Aedes albopictus* eggs ($F_1$) were collected via ovitraps (except some eggs from WM10 were also collected from a tire) between May and June of 2018. The three collection sites from Texas all had varied flora density: WCC was described as an area with low vegetation, WM10 was an area with moderate vegetation and ELG was a natural area with abundant vegetation.

**Supplemental Tables**

Table A.2.1: Final concentrations of bottles used for bottle bioassays and the amounts of working permethrin solutions used to make each concentration. The final concentration of each bottle is after acetone is allowed to evaporate. The 0 μg/bottle is the control without any insecticide.

| Final permethrin concentration (w/o acetone) (μg/bottle) | Volume of 100% acetone added to bottle (μL) | Volume of 1 μg/μL permethrin solution added to bottle (μL) | Volume of 0.1 μg/μL permethrin solution added to bottle (μL) |
|---|---|---|---|
| 0.0 | 1000 | - | - |
| 0.5 | 1000 | - | 5.0 |
| 1.0 | 1000 | 1.0 | - |
| 1.5 | 1000 | 1.5 | - |
| 2.0 | 1000 | 2.0 | - |
| 5.0 | 1000 | 5.0 | - |

Table A.2.2: List of the $LC_{50}$s of all strains and their respective RRs (numbers in bold) and 95% HDIs. (* = the 2 μg bottle of this group only had 2 replicates because there were not enough mosquitoes for a third replicate)

| MEXICO FIELD STRAINS (SELECTION #1) | | | | | | | |
|---|---|---|---|---|---|---|---|
| Strain | Lower $LC_{50}$ | $LC_{50}$ (mean) | Higher $LC_{50}$ | Strain | lower $LC_{50}$ | $LC_{50}$ (mean) | higher $LC_{50}$ |
| ATM $F_{16}$ (1) | 0.27 | 0.55 | 1.2 | ATM $F_{16}$ (2) | 0.68 | 1.12 | 2.00 |
| | | | | | | | |
| Motozintla $F_3$ | 0.59 | 0.96 | 1.61 | G $F_3$ | 1.20 | 2.24 | 4.80 |
| RR & HDI | 2.20 | **1.75** | 1.34 | RR & HDI | 1.76 | **2.00** | 2.40 |
| | | | | | | | |
| Escuintla $F_3$ | 0.45 | 0.74 | 1.27 | F $F_3$ | 0.85 | 1.42 | 2.57 |
| RR & HDI | 1.66 | **1.35** | 1.06 | RR & HDI | 1.25 | **1.27** | 1.29 |
| | | | | | | | |
| Puerto Madero $F_3$ | 0.39 | 0.65 | 1.11 | Huixtla $F_3$ | 0.98 | 1.63 | 2.99 |
| RR | 1.44 | **1.18** | 0.93 | RR & HDI | 1.44 | **1.46** | 1.50 |
| | | | | | | | |
| L $F_3$ | 0.53 | 0.86 | 1.45 | H $F_3$ | 0.86 | 1.45 | 2.56 |
| RR & HDI | 1.96 | **1.56** | 1.21 | RR & HDI | 1.27 | **1.30** | 1.28 |
| | | | | | | | |
| Mapastepec $F_3$ | 0.50 | 0.80 | 1.31 | Huehuetan $F_3$ | 1.00 | 1.86 | 4.15 |
| RR & HDI | 1.84 | **1.45** | 1.09 | RR & HDI | 1.47 | **1.66** | 2.08 |
| | | | | | | | |
| Pijijiapan $F_3$ | 0.53 | 0.87 | 1.48 | | | | |
| RR & HDI | 1.97 | **1.58** | 1.23 | | | | |

(*continued*)

| TEXAS FIELD STRAINS | | | | | | | |
|---|---|---|---|---|---|---|---|
| Strain | lower $LC_{50}$ | $LC_{50}$ (mean) | higher $LC_{50}$ | Strain | lower $LC_{50}$ | $LC_{50}$ (mean) | higher $LC_{50}$ |
| ATM $F_{22}$ (1) | 0.97 | 1.63 | 2.98 | ATM $F_{22}$ (2) | 1.08 | 1.79 | 3.3 |
| | | | | | | | |
| WCC $F_3$ | 2.03 | 3.91 | 9.28 | ELG $F_3$ | 1.93 | 3.45 | 7.12 |
| RR & HDI | 2.09 | **2.40** | 3.11 | RR & HDI | 1.79 | **1.93** | 2.16 |
| | | | | | | | |
| WM10 $F_3$ | 1.24 | 2.23 | 4.75 | | | | |
| RR & HDI | 1.28 | **1.37** | 1.59 | | | | |
| **SELECTED AND NON-SELECTED MEXICO STRAINS** | | | | | | | |
| **SELECTION #2** | | | | **SELECTION #3** | | | |
| Strain | lower $LC_{50}$ | $LC_{50}$ (mean) | higher $LC_{50}$ | Strain | lower $LC_{50}$ | $LC_{50}$ (mean) | higher $LC_{50}$ |
| ATM $F_{18}$ | 0.56 | 0.92 | 1.61 | ATM $F_{20}$ | 1.44 | 2.43 | 4.70 |
| | | | | | | | |
| La Macha $F_5$ | 0.94 | 1.61 | 3.00 | La Macha $F_7$ | 2.46 | 4.95 | 12.92 |
| RR & HDI | 1.68 | **1.75** | 1.86 | RR & HDI | 1.71 | **2.04** | 2.75 |
| **SELECTION #4** | | | | **SELECTION #5** | | | |
| Strain | lower $LC_{50}$ | $LC_{50}$ (mean) | higher $LC_{50}$ | Strain | lower $LC_{50}$ | $LC_{50}$ (mean) | higher $LC_{50}$ |
| ATM $F_{22}$ (1)* | 1.17 | 2.02 | 3.76 | ATM $F_{23}$ | 1.45 | 2.65 | 5.40 |
| | | | | | | | |
| La Macha $F_9$ | 2.33 | 4.57 | 11.84 | La Macha $F_{11}$ | 1.79 | 3.39 | 7.77 |
| RR & HDI | 1.99 | **2.26** | 3.15 | RR & HDI | 1.23 | **1.28** | 1.44 |
| | | | | | | | |
| ATM $F_{22}$ (2) | 1.69 | 3.07 | 6.37 | La Delicada $F_{11}$ | 2.16 | 4.07 | 9.76 |
| | | | | RR & HDI | 1.49 | **1.53** | 1.81 |
| La Delicada $F_9$ | 1.91 | 3.6 | 8.11 | | | | |
| RR & HDI | 1.13 | **1.17** | 1.27 | | | | |

Table A.3.1: Synthetic sequence containing Cysteine mutation (highlighted in pink) used for optimization of the PCR melting curve. Green highlighted nucleotides are sites where we identified synonymous polymorphisms and used the nucleotide that had the highest frequency of occurrence in our *Ae. albopictus* samples that were sequenced. The pink highlighted nucleotide of the underlined codon is the mutation (T→ G) that codes for cysteine in DIIIS6 of the VGSC gene.

| **Sequence of C1534_seq** | **Length (bp)** |
|---|---|
| 5'-GTGGGCAAGCAGCCAATTCGCGAGACCAACATCTACATGTACCTC TACTTCGTGTTCTTCATCATCTGCGGGTCGTTCTTCACCCTTAATCTGT TCATCGGTGTCATCATCGACAACTTCAACGAGCAGAAGAAGAAAGCC GGTGGCTCGCTGGAAATGTTCATGACGGAGGATCAGAAAAAGTACT ACAACGCAATGAAAAAGATGGGCTCGAAGAAGCCG-3' | 222 |

Table A.3.2: Mean values and standard errors (SE) of each enzyme activity that was assayed for the three *Ae. albopictus* strains (ATMNJ95, La Delicada (No Selection), La Macha (Selection)) and the three *Ae. aegypti* strains (New Orleans, Susceptible Vergel (Vergel-S), Resistant Vergel (Vergel-R)). Note: the number of observations (n) for each strain within each enzyme assay was 40, except for the PNPA assays of La Macha and Vergel-R, where n=39 due to the removal of samples that were extreme outliers and had $R^2$ values < 0.5 when correlating obtained and expected results.

| | ***Aedes albopictus* Strains** | | | ***Aedes aegypti* Strains** | | |
|---|---|---|---|---|---|---|
| **Enzyme assayed** | ATMNJ95 | La Delicada (No Sel.) | La Macha (Sel.) | New Orleans | Vergel (Sus.) | Vergel (Res.) |
| Acetylcholin-esterase (% inhibition) | 86.21 (±1.08) | 91.01 (±0.65) | 87.53 (±0.94) | 90.21 (±0.75) | 91.82 (±0.65) | 82.56 (±0.73) |
| Cytochrome P450s (µg/ptn) | 0.17 (±0.0033) | 0.20 (±0.0032) | 0.20 (±0.0046) | 0.13 (±0.0019) | 0.15 (±0.0025) | 0.15 (±0.0030) |
| Alpha-esterases (nmol naphtol/mg ptn/min) | 11.12 (±0.19) | 11.75 (±0.29) | 10.78 (±0.17) | 8.26 (±0.13) | 9.37 (±0.24) | 8.83 (±0.20) |
| Beta-esterases (nmol naphtol/mg ptn/min) | 8.10 (±0.20) | 8.19 (±0.36) | 6.85 (±0.18) | 4.19 (±0.17) | 6.23 (±0.29) | 4.45 (±0.18) |
| Glutathione *S*-transferases (mml/mgprot/ min) | 0.77 (±0.029) | 0.86 (±0.050) | 0.68 (±0.035) | 1.00 (±0.035) | 0.98 (±0.048) | 0.87 (±0.037) |
| P-nitrophenyl acetates (Δ ABS/mg ptn/min) | 2.16 (±0.057) | 2.58 (±0.11) | 1.76 (±0.080) | 2.31 (±0.14) | 2.46 (±0.17) | 2.88 (±0.15) |