DISSERTATION

EVOLUTION OF MUTATIONS ASSOCIATED WITH PYRETHROID RESISTANCE AND THE REVERSAL OF RESISTANCE IN *AEDES AEGYPTI*

Submitted by

Farah Zamira Vera Maloof

Department of Microbiology, Immunology and Pathology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2019

Doctoral Committee:

Advisor: William Black

Janet McAllister
Brian Foy
Louis Bjostad
Ashley McGrew

ABSTRACT

EVOLUTION OF MUTATIONS ASSOCIATED WITH PYRETHROID RESISTANCE AND THE REVERSAL OF RESISTANCE IN *AEDES AEGYPTI* (Linnaeus)

Worldwide vector control has been relying heavily on pyrethroid insecticides to reduce *Aedes aegypti* Linnaeus populations. Pyrethroids are relatively inexpensive, have low vertebrate toxicity, and have been efficient in reducing mosquito populations. Constant use of pyrethroid insecticides, however, has driven mosquito populations to develop resistance over time. In this dissertation, we have tracked the evolution of three mutations in the voltage gated sodium channel (vgsc) that are associated with pyrethroid resistance *Aedes aegypti* populations in Mexican. These are 410, 1,016 and 1,534, corresponding to the position of amino acid substitutions in the vgsc. A valine at locus 410 (V410) confers susceptibility, while leucine (L410) confers resistance. A valine at locus 1,016 (V1,016) confers susceptibility, while isoleucine (I1,016) confers resistance. A phenylalanine at locus 1,534 (F1,534) confers susceptibility, while cysteine (C1,534) confers resistance. We performed a linkage disequilibrium analysis of the three mutations in Mexican collections from 2000–2016.

In the first study, a linkage disequilibrium analysis was performed on I1,016 and C1,534 in *Ae. aegypti* collected in Mexico from 2000–2012, to test, in natural populations, for statistical associations between segment six (S6) in domains II and III of the vgsc. We estimated the frequency of the four di-locus haplotypes in 1,016 and 1,534: $V_{1,016}/F_{1,534}$ (susceptible), $V_{1,016}/C_{1,534}$, $I_{1,016}/F_{1,534}$, and $I_{1,016}/C_{1,534}$ (resistant). The susceptible $V_{1,016}/F_{1,534}$ di-locus haplotype went from near fixation to extinction, and the resistant $I_{1,016}/C_{1,534}$ di-locus haplotype

increased in all collections from a frequency near zero, to frequencies ranging from 0.5–0.9. The $V_{1,016}/C_{1,534}$ di-locus haplotype frequency increased in all collections until 2008. After this year, the frequencies in two collections began to decrease, likely due to the fact that the $I_{1,016}/C_{1,534}$ di-locus haplotype frequency increased in all collections. However, the $I_{1,016}/F_{1,534}$ di-locus haplotype was rarely detected; for instance, it reached a frequency of only 0.09 in one collection and subsequently declined.

Pyrethroid resistance in the *vgsc* gene appears to require the sequential evolution of two mutations. The $I_{1,016}/F_{1,534}$ di-locus haplotype appears to have low fitness, suggesting that I1,016 was unlikely to have evolved independently. Instead the C1,534 mutation evolved first but conferred only a low level of resistance. I1,016 in S6 of domain II then arose from the $V_{1,016}/C_{1,534}$ haplotype and was rapidly selected because double mutations confer higher pyrethroid resistance. This pattern suggests that knowledge of the frequencies of mutations in both S6 in domains II and III are important to predict the potential of a population to evolve kdr. Susceptible populations with high $V_{1,016}/C_{1,534}$ frequencies are at high risk for kdr evolution, whereas susceptible populations without either mutation are less likely to evolve high levels of kdr, at least over a 10 year period.

In the second chapter we describe a novel replacement V410L that was initially detected in a pyrethroid resistant insectary strain from Brazilian *Ae. aegypti* populations. We screened V410L in 25 *Ae. aegypti* historical collections from Mexico. The first heterozygote appeared in 2002, and frequencies have increased in the last 16 years, along with I1,016 and C1,534. L410 showed a strong association between 1,534 and 1,016 mutations. Individuals with the triple homozygote resistant genotype had higher survival after pyrethroid exposure, 96% of the alive

individuals had the triple homozygote resistant genotype after permethrin and 76% after deltamethrin treatment.

The purpose of insecticide resistance management strategies is to minimize the selection for resistance to any one type of insecticide, or to help regain susceptibility in insect populations in which resistance has already arisen. A key component of resistance management assumes that there will be a negative fitness associated with resistance alleles, so that when insecticides are removed, resistance alleles will decline in frequency.

In the third chapter we tested for the loss of pyrethroid resistance from eight field populations of *Ae. aegypti*, (six field collections from or near the city of Merida, and two collections from Tapachula and Acapulco in southern Mexico) to assess variation in the rate of loss of pyrethroid resistance. Collections were maintained for up to eight generations after pyrethroids were discontinued. We recorded changes in the frequencies of two kdr mutations, I1,016 and C1,534, and the analysis of resistance ratios (RR) with permethrin (pyrethroid type 1) and deltamethrin (pyrethroid type 2). In generations F_3, F_6, and F_8, we also evaluated fecundity to test for parallel changes in a fitness trait during the eight generations. This was analyzed because a negative association between resistance and fecundity had previously been described in two studies [1, 2]. We demonstrate that the frequency of the *Ae. aegypti* pyrethroid resistance alleles I1,016 and C1,534 decline when pyrethroid pressure is removed in the laboratory; however, the pattern of decline is strain dependent. In agreement with earlier studies, fecundity was negatively associated with the frequency of resistance alleles.

ACKNOWLEDGEMENTS

"If I have been able to see farther than others, it was because I stood on the shoulders of giants"
Isaac Newton

While working in Mexico several years ago, I was offered an amazing opportunity from the National Institute of Health Fogarty International Center to come to the United States to do vector control research with Dr. William Black at Colorado State University. After working with Dr. Black for four months, he offered a position in his lab as a PhD student. However, in order to do this, I had to make a difficult decision: Do I stay and have a stable job in Mexico where my whole my family lives, or do I take a chance on a new experience and push myself out of my comfort zone in order to achieve a bigger dream of mine? In the end, I took a chance and came to Colorado. Despite all of the challenges I had to face, such as the language and cultural barriers of being in a foreign country, my dream came true. I was fortunate to have an amazing network of colleagues that helped me to expand my capabilities and resilience and I would like to acknowledge and thank them. First, this work would not have been possible without Dr. Black´s guidance and mentorship. I still have so much to learn from him. He has the capacity of seeing things from a different perspective than others. He challenges me to stretch the boundaries of my capabilities and if I am as successful in my career as I hope to be, it will be in large part because of him.

I would also like to thank Dr. Karla Saavedra for being a tremendous teacher and a person that I can always count on. Susan Rogers and Heidi Runge were the best administrators and academic support coordinators; they would always answer all of my questions and always found a way to help me when I needed it the most. I also want to thank my past and present lab

mates who have taught me so much and have been wonderful friends to me: Miguel Moreno, Abdiel Martin-Park, Armando Elizondo, Selene Garcia-Luna, Karen Fleming, Saul Lozano, Nick Arthur, Lourdes Talavera, and Sheena Francis.

I want to thank the undergraduate students that helped me complete many, many DNA extractions: Connor Hendrich, Paisley Byrnes, Ashley Janich, Jamaine Holt, Kyra Headrick, and Alana Kim. Additionally I would like to thank Ashley Janich for her helping me edit my written work and for being an excellent lab mate to work with.

I want to thank my friend Liz Parra, who is my confidant and was always there to listen when I needed someone to talk to. We had many long talks about life and our shared experiences along this journey. She was always encouraging me to be the best person I could be. I also owe thanks to my friends Reuben Addo, Dhavhita Gwenzi, Diedo Mulamba, Lety Maldonado for always having positive attitudes that inspire me. Natalia Voge for always rooting for me. Ana Carolina, Claudia, Fredy, Sol, Martin, Cristian for always making me laugh, sing and to celebrate with. I want to thank my friend Jhibran Ferral-Piña, whom I can always count on no matter how much distance separates us. I want to thank Alma Lopez and Paco Solis for being amazing lab mates and dear friends, always al pie del cañon, and for being hard working people.

I want to give thanks to AnaMario Benites and the family of Silva-Tzompa (Adriano and Zitely), Rodriguez-Penilla (Americo and Paty), Lozano-Saavedra (Saul and Karla), and Gutierrez-Bandon (Enrique and Erika) for allowing me to stay in your homes when I needed it the most. I am forever grateful to you all!

And thank you, Dr. Kristina Quynn, for teaching me how to make writing more frequent, less stressful, and actually enjoyable! I truly appreciated my time working with you. I have

improved my writing and my time management skills with your guidance and by following your example.

I want to say thank you to our collaborators from Mexico from whom we obtained all our Mexican mosquito strains: Dr. Americo Rodriguez of Centro Regional de Investigacion en Salud Publica (CRISP) for sending us mosquito eggs from Chiapas, Felipe Dzul of Centro Nacional de Programas Preventivos y Control de Enfermedades (CENAPRECE) for sending mosquito eggs from Guerrero, and finally, Dr. Pablo Manrique and Dr. Abdiel Martin of the Universidad Autonoma de Yucatan (UADY) for sending mosquito eggs from the state of Yucatan.

I also want to thank to my committee members: Janet McAllister, Ashley McGrew, Brian Foy, and Louis Bjostad for their support and input on my project.

On this adventure, I not only got to learn and develop more professional skills and knowledge, but I also I feel so lucky for having met all these amazing people. The emotional and professional support from them helped make this work a reality.

Thank you all so very much!

DEDICATION

This work is dedicated to my family, who are my biggest fans! Especially to my parents, Alfredo Vera Garcia and Teresa Maloof Arzola who always have been my biggest supporters in life. Thank you for all your love! I am forever grateful to you both for giving me the opportunity to forge my own path and for supporting me, both emotionally and financially, when I needed it the most. This is also dedicated to my sister Talye Karime, who kept me company by skyping with me when I needed to work late at the lab, and to my sister Karla Munira, my brothers Alfredo Roberto and Farid Salomon who are my rocks and who always help me when I am feeling down. They are my inspiration and my best friends.

I also dedicate this work to my aunts Irma, Munira, Dumy, Nieves and Cuca who are examples of what it means to be great Mexican women, luchonas. They have always been grateful for the lives they've been given, and radiate positive, cheerful energy into the world. This work is also for my uncles, Arturo y Jose Luis for taking great interest in my research, and for all of my extended family; too-many-to-name uncles, aunts, cousins, nieces, nephews. Thank you for being my family, for better or worse.

This work is also dedicated to my childhood friends Elizabeth Guerson and Cynthia Betacourt. Elizabeth, I am sorry I missed your wedding, and Cynthia, I am sorry I was not able to be there for your son's baptism. But I was with you in spirit while I was working on my research and if I could have been there for you both, you know I would have! And lastly, to my friends from college, Sarai Olivier and Selene Cruz-Garcia, for being a huge part of my cheerleading team.

¡Los amo mi hermosa familia!

TABLE OF CONTENTS

Chapter 1: Literature Review

Aedes aegypti as a vector of human diseases in the Americas

Diseases that are caused by microorganisms transmitted between animals and humans are referred to as **zoonotic diseases** and play an important role in introducing pathogens to human populations. For instance, 61% of the infectious agents that cause diseases in humans are considered to be zoonotic [3, 4]. According to World Health Organization an **emerging zoonosis** is a "newly recognized or newly evolved, or that has occurred previously but shows an increase in incidence or expansion in geographical, host or vector range". Interestingly, 76% of viruses are more likely to emerge in the transmission routes of zoonotic pathways [3]. One example of an emerging zoonosis is the arrival of Zika virus in the Americas. This and other mosquito-borne diseases have increased concern about the impact of mosquitoes on human health. **Vector-borne diseases** are responsible for 22.8% of emerging infectious diseases (EID), globally, and 25.4% of the EID are viral pathogens [4]. Historically, mosquitoes spread many viruses to human populations all around the world, and their role in disease transmission is a huge concern to public health.

During human history, several epidemics involving mosquitoes have struck human populations around the world. Mosquitoes are vectors of pathogens that cause human diseases that have had a huge impact on global economies and public health. In the Americas, there are numerous records of illness where mosquitoes are involved. For instance, yellow fever (YF) caused the deaths of thousands of people [5]. Fortunately, there is currently an YF vaccine available; therefore, cases tend to be more geographically contained. However, for other

arboviruses such as dengue, chikungunya, or Zika, there are still significant burdens on public health since there is no effective vaccine or treatment available to the general public.

The genus *Aedes*, is responsible for transmiting Zika, chikungunya, dengue and YF viruses. *Aedes aegypti* and *Aedes albopictus* are the main species. *Aedes aegypti* is commonly known as the "yellow fever mosquito", and is well known because of the large numbers of pathogens that it transmits. *Aedes aegypti* originated in Africa and likely arrived in the New World on ships with the slave trade and human travel. DNA sequencing and large-scale single nucleotide polymorphisms (SNP) analysis provide evidence that supports this hypothesis [6, 7]. *Aedes aegypti* arrived soon after Europeans first arrived to the New World, along with yellow fever virus. The earliest epidemic possibly appeared in the Caribbean islands in 1647 [5], however, Spanish colonists in 1648 were the first to record an outbreak of YF in the Yucatan peninsula of Mexico, which the locals called *xekik*, meaning black/blood vomit (i.e. indication of severe YF) in Mayan [8]. Currently, an effective prophylactic vaccine is available for YF that is effective in reducing distribution of the disease. However, in December 2016, a YF outbreak was detected in Brazil, followed by isolated cases in Colombia, Ecuador, Peru, Bolivia, and Suriname, in 2017 [9].

Dengue virus occurs in tropical and subtropical regions, the most recent estimation for dengue is 390 million infections per year of which 96 million are symptomatic [10]. It is estimated that 14% of worldwide infections occur in the Americas and more than 50% of these are in Brazil and Mexico [10]. Moreover, chikungunya virus (CHIKV) was documented in December of 2013. The first outbreak of CHIKV in the Americas was confirmed in the Caribbean island of St. Martin as autochthonous. Since then, local transmission has been

reported in more than 45 countries in the Americas [11]. According to the Pan American Health Organization, from the time of introduction through epidemiological week 51 in 2017, more than 2 million suspected or confirmed cases of chikungunya were recorded. Thus far, a total of 524 deaths have been attributed to CHIKV in the Western Hemisphere. There have not been any reports of autochthonous infections in Canada, Cuba, Chile and Uruguay.

The most recent virus transmitted by *Aedes* in the Americas is Zika virus (ZIKV). ZIKV infections were first detected in Brazil [12] followed by Colombia, the Dominican Republic, Mexico, Paraguay and El Salvador. In the Americas, 806,928 autochthonous (suspected or confirmed) cases of Zika and 20 deaths associated with the disease were reported from 2015 to 2017 [13]. Beside the symptoms of this disease, there are other health problems associated with ZIKV infection; an increase in birth defects has been reported, including microcephaly in the developing fetus associated with ZIKV infections. Starting in 2018, the Pan American Health Organization reported 3,720 confirmed congenital syndrome associated infections of this flavivirus. Most (79%) of these cases were located in Brazil. There was also a correlation between Guillan-Barré syndrome and the presence of ZIKV. ZIKV has been reported to be transmitted from person to person through sexual intercourse [14]. The Centers for Disease Control and Prevention has reported 52 sexually transmitted cases in the US from 2015 through April 4th 2018 [15]. In other territories, it is not possible to determine whether infection occurred due to mosquito bite or sexual transmission, due to the high number of cases of ZIKV. Bermuda, Canada, Chile and Uruguay have only reported imported cases [13].

Identification and biology of *Aedes aegypti* (Linnaeus, 1762)

The most consistent morphologic characteristic of *Ae. aegypti* mosquitoes is the distinctive scale pattern on the dorsal side of the thorax, consisting of two silver straight lines surrounded by curved lyre-shaped lateral lines, contrasting with the general covering of narrow dark scales. It is believed that the ancestor of the domestic form of *Ae. aegypti* is from sub-Saharan Africa and can still be found there today, it is known by the subspecies name *formosus* [6, 16-18]. This subspecies lives in forests, lays their eggs in tree holes and prefers non-human blood. Morphologically, this form is much darker in color than the form adapted to human habitats, it also has a different scaling pattern than the domestic form [16, 19]. It has been observed that the behavioral traits of subspecies *aegypti* and *formosus* are associated with urban and sylvan breeding respectively [17, 20]. As previously mentioned, it is almost certain that *Ae. aegypti* arrived to the New World on ships where conditions may have selected for the domestic type [6, 17].

Aedes aegypti is an urban and domestic mosquito which means that it often lives in cities and in or near to homes. This mosquito can mature, from egg to adult in 7-10 days, whereas adult mosquitos generally live 4-6 weeks. It is the female who is responsible for the transmission of viruses because it needs blood to nourish its developing eggs. The male does not feed on blood. Females feed every 3-4 days; however, if they fail to ingest enough blood, they continue to feed on additional hosts. The mosquito is most active in the early morning and around dusk, making these the periods of highest risk for bites. However, females that need to continue feeding will look for a blood source at any time. After feeding, the females lay eggs every 3-4 days in different containers, ensuring that some of its offspring will survive predators, and making mosquitos more difficult to control. Females lays their eggs in artificial containers holding water

(drums, barrels, vases and tires mainly) in and around houses, schools and workplaces. That is why it is important to dispose of unused containers in and around the home and in places where people get together for long periods of time (e.g. schools), and protecting containers that store water (i.e. sealing them or treating them with chemical or biological products). Females can lay up to 400 eggs in the course of their lives. The number of eggs laid in each gonotrophic cycle depends on the age and size of the female and the amount of blood ingested. *Aedes aegypti* eggs can resist dry environmental conditions for more than a year, desiccation tolerance is one of the most important strategies the species employs for survive and spread. That is why properly cleaning the surfaces of drums and barrels is necessary to control this vector [21]. In general, *Ae. aegypti* has a short lifetime flight range, not exceeding 150 meters because homes provide food and breeding sites. However, mosquitoes have been observed to fly for as far as 400 meters in search of food [22].

Arthropo's nervous system

My dissertation is focuses on the genetics of pyrethroid resistance and how pyrethroids act on the nervous system. A neuron consists of a **soma** (cell body), a **nucleus**, **dendrites and axon.** Dendrites receive the signals from others neurons and transmits to the soma and the axon, which transmit the impulses away from the soma. The junction between one neuron and the next is called the **synapse**. Cations concentrate outside the neuronal cell through proton pumps driving then out of the cell creating a voltage differential across the membrane known as the **resting membrane potential**. Inside the cell, in a resting state, the charge is typically between – 70 and – 90 mV. That difference in electrical potential, or polarization, is maintained by ion (sodium/potassium) pumps [23, 24].

When there is a stimulus from the environment, the sensory receptors convert this potential energy into electrical energy. The stimulus or signal causes the dendrites of the sensory receptor to undergo a depolarization (reduction in voltage due to a flow of cations inside the cell, between -50 to +30 mV). This depolarization occurs progressively along the axon and is known as an **action potential** [23]. The depolarization continues until it reaches the synapse. When depolarization arrives at the presynaptic membrane, ion channels open, allowing cations to enter the neuronal cell which stimulates synaptic vesicles to fuse with the membrane, releasing neurotransmitters into the synaptic cleft. Once the neurotransmitter crosses the synaptic cleft, it binds to specific receptors on the postsynaptic membrane, inducing conformational changes that alter the membrane permeability and inducing subsequent depolarization of the second neuron (or a contraction if it terminates in a muscle cell).

Many different neurotransmitters, neuromodulators, and neuropeptides are involved in nervous transmission, either stimulating or inhibiting the postsynaptic membrane. For example, acetylcholine (ACh) is the most common excitatory neurotransmitter while γ-aminobutyric acid (GABA) is an inhibitory neurotransmitter. Enzymes within the synaptic cleft degrade the neurotransmitters after they are bound, and allow some of these materials to be recycled to the presynaptic neuron for resynthesis of neurotransmitters in the vesicles. This degradation also prevents the neurons from being continually activated by a stimulus that is no longer present, and opens the receptor to stimulation by subsequent release of neurotransmitter. Some insecticides act on the enzymes or their receptor. For instance, organophosphate and carbamate insecticides target the enzyme acetylcholinesterase and prevent the degradation of acetylcholine at the synapse while organochlorides target GABA receptors [25].

Voltage Gated Sodium Channel

Voltage Gated Sodium Channel (vgsc) proteins are located all along the axon. The channel is responsible for the initiation and propagation of action potentials in the nervous system and other excitable cells. The vgsc is a transmembrane protein that crosses the entire cell membrane from inside to outside the cell. It forms a pore that is highly selective for sodium ions. The vgsc had two subunits: α and β. The **α-subunit** is the main structure that forms the actual pore; whereas, the β-subunit is an accessory segment. *Aedes aegypti* have one α-subunit gene, unlike humans which have nine [26, 27]. The α-subunit consists of four homologous **domains (I-IV)** and each domain has six **segments/helices (S1-S6)**. The homologous domains have a clockwise arrangement in the membrane which forms the pore (**Figure 1.1**) [28].

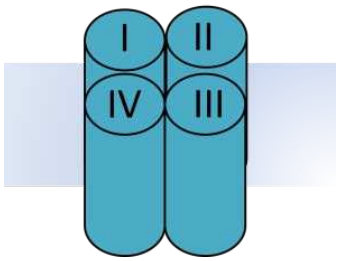


Figure 1.1. Clockwise arrangement of the four domains in the α-subunit of the sodium channel.

The extracellular linker between S5 and S6 at each domain is known as P-loop and form the **selectivity filter** for sodium ions. The P-loop along with the S5 and S6 segment of the four domains forms the actual pore, also known as a P-region. Each S4 segment consists of repetitive positively charged amino acids follow by two hydrophobic residues and along with the S1-S3 constitute the **voltage sensor**. The S6 segments meet together at the base of the pore, at the cytoplasmic end, which forms the **activation gate** [29]. The intracellular linker between

segment S6 of domain III and segment S1 of domain IV constitutes the **inactivity gate** (**Figure 1.2**).

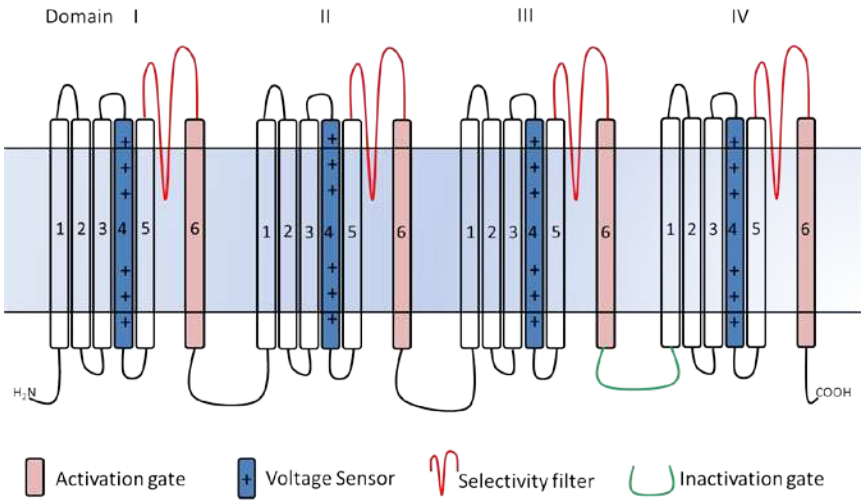


Figure 1. 2. Structure of the voltage-gated sodium channel. Segment 1-4 form the voltage sensor, S4 will move outwards and inwards (blue) in depolarization and repolarization, respectively. Linkers 5-6 constitute the filter for Na^+ ions (red), segments S6 form the activation gate (pink). Linker between domain III and IV constitute the inactivation gate (green).

The conformation of S4 changes as depolarization progresses. As a result, the channel opens by the opening of the activation gate, a process called **activation state**, which allows the influx of Na^+ ions. Milliseconds after activation, the inactivation gate physically closes the pore, obstructing the influx of sodium, a process known as **inactivation state**. After repolarization, the activation gate returns to the closed state known as **deactivation.** The pore is closed by the activation gate and the blocking of the inactivation gate. Within a refractory period of a few milliseconds, conformational changes of the activation gate force the inactivation gate to pull out from the pore. Then, the pore reopens and it returns to the closed resting potential state. The vgsc is ready for the next firing of the action potential [30].

Vector control and mode of actions

Even though there is a vaccine available for yellow fever and a phase III trial vaccine is being tested for dengue fever, there are other diseases transmitted by *Ae. aegypti* which have no effective drug treatment or vaccines available (e.g. Zika and chikungunya). Therefore, the most effective way to control outbreaks and stop dispersion of those diseases is by management of the vector populations. There are many methods that can be used to control *Ae. aegypi*. The choice of which method to use depends on various factors. Every case has unique environmental conditions, as well as the involvement of the community with their public health department, and also the resources available, and/or insecticide resistance status.

Chemical used in *Aedes aegypti* control and mode of actions

Those insecticides that target different pathways of the insect's nervous system are the most common and effective methods to control mosquitoes because results can be seen in a short period of time. Organophosphates, carbamates, organochlorines and pyrethroids are the four main groups of insecticides used worldwide. However, there are others chemicals that target different pathways of insect development, like blocking the formation of chitin, or mimicking, or blocking the production of the juvenile hormones. Other chemicals that block respiration are also used in some conditions.

Insecticide acting on sodium channels

Insecticides, such pyrethroids and DDT, act on the sodium channels. These insecticides bind to the open state of the channel since they have a higher affinity to the open stage. These insecticides obstruct and delay the inactive stage, prolonging the opening of the channel, and

allowing more Na^+ ions to enter the neuronal cell [31]. Repetitive discharge of the action potential arises from a single stimulus resulting in hyperactive tremors following paralysis, and ultimately death of the insect [30, 32, 33].

Indoxacarb and metaflumizone are new classes of insecticides that also target the sodium channel; however, these insecticides have a higher affinity for the inactive state of the channel. Instead of prolonging the open state, they inhibit the sodium current. These types of insecticides are known as sodium channel inhibitors (SCIs) [34].

Dichlorodiphenyltrichloroethane (DDT)

DDT (organochlorine) was the first of the modern insecticides, synthesized in 1873, but it was not until 1939 that Dr. Paul Müller discovered the insecticide activity of DDT. Although, DDT has been banned in USA and other countries since 1972 due to its persistence in the ecosystem, and bioaccumulation in the fats of vertebrates. However, DDT is still very important for malaria control in India and Africa. The DDT´s lethal dose that kills 50% (LD_{50}) in rats is 250 mg/kg [23].

Docking of DDT molecules into the mosquito's sodium channel has been investigated by computer modeling. This approach has suggested that two DDT molecules are needed to bind simultaneously to two receptor sites of the vgsc to exert the insecticidal effect of DDT. The receptor sites are the same in the pyrethroid receptors sites, PyR1 and PyR2 [35].

Pyrethroids

Pyrethroids are the synthetic forms of the natural insecticides known as pyrethrins. This natural insecticide is found in pyrethrum, an extract of dried flowers from the *Chrysanthemum*

genus [36]. In the past five decades, the use of pyrethroids have increased worldwide. This is due, in part, to their high selectivity, high efficiency as an insecticide, low toxicity to mammals, and low environmental hazards compared with other classes of insecticides [37]. Pyrethroids are widely used in agriculture, homes, and in vector control for public health. Pyrethroids represent 17% of the global market of insecticides [38], and in Mexico, they are the most common insecticide, representing the 31.5% of the products used for vector control [39] (**Figure 1.3**).

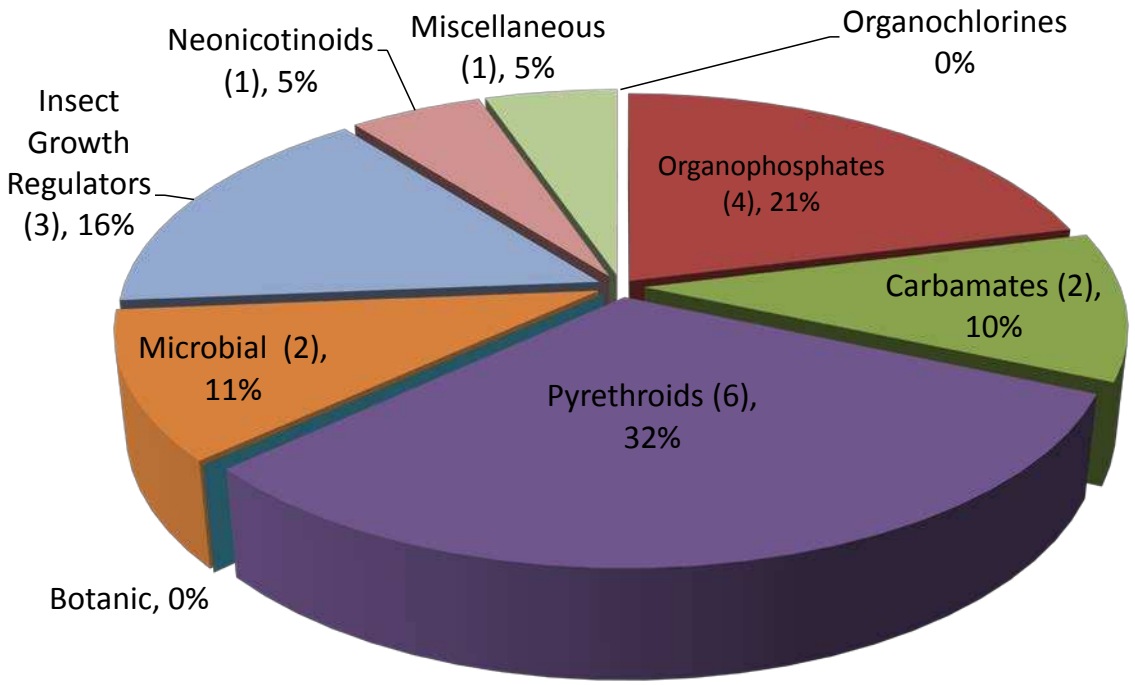


Figure 1. 3. Classes of insecticides recommended for vector control in Mexico by CENAPRECE 2018.

Pyrethroids are classified as two different types, type I and II, based on their effects on sensory neurons in cockroaches [40]. **Type I pyrethroids**, lack an α-cyano group on the phenoxybenzyl moiety (e.g. permethrin, resmethrin, teramethrin, allethrin, bifenthrin, metofluthrin, prallethrin, and *d*-phenothrin) and include the non-ester pyrethroid etofenprox. Type I compounds, are often associated with whole body tremors, convulsions, twitching, coma

and death in rats. It is also classified as T (tremor) intoxication syndrome [40-43]. **Type II pyrethroid**, have an α-cyano group (eg. cypermethrin, fenvalerate, tralomethrin, esfenvalerate, deltamethrin, λ-cyhalothrin, cyfluthrin). Type II pyrethroids are classified as a choreoathetosis with salivation (CS) intoxication syndrome since intoxication symptoms consist of salivation, whole body tremors and progressive writhing convulsions (choreoathetosis) in vertebrates [40-43].

Pyrethroids bind to two receptor sites in the vgsc, PyR1 and PyR2. Both contain lipids that are exposed in the open state of the sodium channel. PyR1 is located along the linker connecting segment S4 and S5 of domain II (IIL4-5), segment S5 in domain II (IIS5), and segment S6 in domain III (IIIS6) in the open stage [44]. PyR2 is located along linker connecting S4 and S5 segments in domain I (IL4-5), the segment S5 and segment S6 of domain I (IS5 and IS6, respectively) and segment S6 of domain II (IIS6) [29] (**Figure 1.4**).

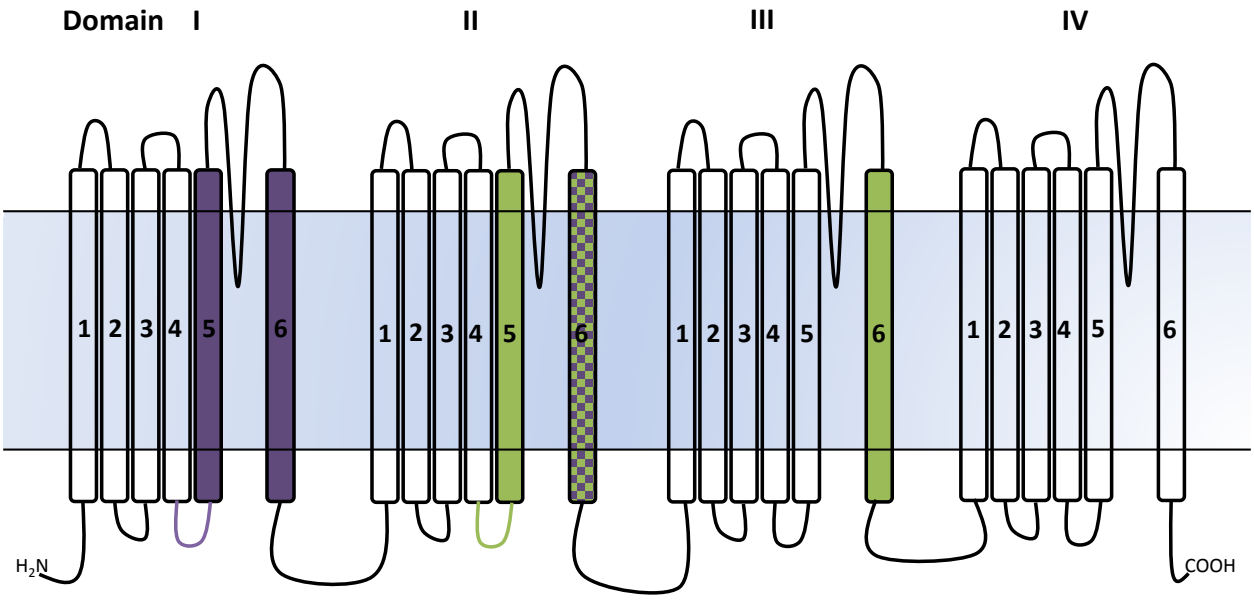


Figure 1.4. Two receptor site of pyrethroids in the voltage-gated sodium channel. PyR1 (Green) and PyR2 (Purple).

Inhibition of acetylcholinesterase (AChE)

Acetylcholine, a neurotransmitter, is released into the synaptic cleft and binds to the receptor of the postsynaptic membrane, thus generating a new action potential in the receiving cell. AChE is located in the synapses and is the enzyme that removes the excess of acetylcholine in the synapses to avoid repetitive excitation by the presence of acetylcholine [23].

Insecticides such as carbamates and organophosphates (OPs) act on the central nervous system of insects, interfering with the flow of nerve impulses by inhibiting AChE. OPs and carbamates bind into the active site of the AChE by mimicking the structure of the acetycholine (ACh), preventing degradation of Ach and therefore resulting in the accumulation of ACh in the synapse and excessive neuroexcitation.

Carbamates

Carbamate insecticides are esters of carbamic acid. A carbamate is generated by replacing the hydrogen atoms in the molecule with aliphatic or aromatic radicals. Carbamates constitute 6% of the global insecticide market [38] and 11% of the products used against vector insects in Mexico [CENAPRECE, 39] (**Figure 1.3**). Bendiocarb and propoxur are the most common carbamate insecticides used in public health to control mosquitoes [45]. Carbamates have been used as indoor residual sprays [45]. In rats the oral LD_{50} is 95-104 and 40-156 mg/kg for propoxur and bendiocarb respectively [23].

Organophosphates

Organophosphates (OPs) were produced during World War II. These included nerve gases such as sarin, soman, and tabun. OPs are a large class of insecticide and highly toxic. They

are phosphoric acid derivatives. The hydrogen atoms of phosphoric acid can be replaced with organic radicals, such as methyl, ethyl, or phenyl groups. Additional modifications involve oxygen atoms being replaced by sulfur, carbon, or nitrogen [23].

Organophosphates capture 13% of the global insecticide market [38], and constitute 21% of the recommended products to fight insect vectors of diseases for 2018 in Mexico [CENAPRECE, 39] (**Figure 1.3**). Chlorpyrifos, temephos, and malathion are examples of organophosphates and have oral LD_{50} in rats of 96-270, 4,204-10,000, 900-5800 mg/kg, respectively [23].

OPs are more toxic to humans than carbamates. The decarbamylation of acetylcholinesterase is faster, requiring minutes, while the dephosphorylation of AChE in the presence of organophosphates is slow, taking days. OPs-AChE binding is considered to be irreversible (non-competitive inhibition), while carbamates may unbind the receptor site, leaving the active site available for ACh to bind. Carbamates are known as reversible acetylcholinesterase inhibitors due to the competition of the carbamate and ACh for the AChE's active site [23]. AChE and ACh are common in the central nervous system of invertebrates and vertebrates; therefore, these two classes of insecticides are toxic to all animals, including humans.

Insect Growth Regulators

Insect growth regulators (IGRs) are a newer class of compounds, with a different mode of action from those of conventional insecticides that act as neurotoxins or behavior modifiers. These types of chemicals interrupt insect growth and development, eventually resulting in death. Since, IGRs affect insect growth, they are applied at the larval stage; they are not affective

against adult insects. IGRs are more selective, and do not have adverse effects on humans, wildlife and the environment. Based on differences in their chemical structure, there are currently five groups of IGRs that exhibit different modes of action [23]. **Chitin biosynthesis inhibitors** prevent the proper formation of the exoskeleton when the insect molts. The insecticide acts in the last step of chitin synthesis, affecting resistance and elasticity of the endocuticle; thus, the cuticle is unable to tolerate molting, thereby resulting in death [23]. In addition, they affect embryo development resulting in unviable eggs (e.g. benzoylphenylureas, thiadiazines) [46, 47]. Other IGR insecticides include **molting disruptors** such as cyromazine, which alter cuticle sclerotization. This stiffening of the cuticle does not allow for expansion necessary during the molting process [23]. **Juvenile hormone mimics** have a different mode of action than the IGRs. In insects, the main role of juvenile hormone (JH) is to maintain the ecdysone (steroid hormone) at levels that maintain larval and nymphal development, by preventing premature metamorphosis and ensuring proper larval growth. In insects, JH is present in higher levels in immature stages and decreases as the larva proceeds through subsequent instars. Thus, JH is undetectable the last` larval instar, so that adult metamorphosis can occur. Methoprene or pyriproxyfen mimics juvenile hormone and often is very toxic when applied in early larval instars, resulting in inhibition of embryogenesis, metamorphosis and morphological abnormalities in eclosed adults. Finally, **ecdysone receptor agonists** bind to specific ecdysone receptors, inducing precocious molting and result in larval death (i.e. Diacylhydrazines) [23]. **Table 1.1** lists examples of insecticides in each group and their mammalian oral LD_{50}. In Mexico, the IGR juvenoid, methoprene, and the benzoylphenylurea novaluron are currently being used for mosquito control [39].

Table 1.1. Insect growth regulators (IGRs) and an insecticide example of each group type of IGR, their corresponding LD50 and examples of insects that are on product labels. The different colors correspond different mode of action of the IGRs, Yellow=Juvenile hormone mimics, Green=Inhibitors of chitin biosynthesis, White=Ecdysone receptor agonists, Orange=Molting disruptors.

Insect Growth Regulators	Insecticide	Oral LD_{50} in rats (mg/kg)	Examples of insects on product labels
Juvenoids	Methoprene	34,600	Mosquitoes, fleas, horn flies
	Pyriproxyfen	>5000	Houseflies, mosquitoes, fleas
Benzoylphenylureas	Diflubenzuron	4640	Mosquitoes, flies, lepidopterans
	Teflubenzuron	>5000	Lepidopterans, coleopterous, fly
	Novaluron	>5000	Whiteflies, lepidopterans
Thiadiazines	Buprofezin	2198	Mealybugs, rice planthoppers
Diacylhydrazines	Tebufenozide	>5000	Lepidopterans
	Halofenozide	>5000	Grubs, soilborne turf pests
Triazines	Cyromazine	3387	Leafminers

Oils

Oils have been applied to the surface of water to kill aquatic insects that depend on oxygen at the water surface. Thus, oils have low species specificity. Oils acts physically not chemically by spreading over water to form an ultra-thin film that lowers water surface tension and prevent the respiratory siphon from opening. A monomolecular layer prevents proper orientation of mosquito larvae and/or pupae to the surface, and inhibits the opening of their respiratory siphon's hairs, resulting in suffocation and finally death [48, 49].

In the past, oils such as diesel and kerosene have been used for killing mosquito larvae due to their low cost. However, they are no longer used because of their low specificity and slow biodegradation. Currently, products that are more specific (e.g. to mosquitoes' larvae) and environmentally friendly, made from plant oils, have been developed (e.g. Agnique).

Biological control

Predation, parasitism and transgenic organisms are the main biological options to mitigate the vectors of pathogens. Use of biological control requires time to see results. In an outbreak, to reduce the target population of infected immediately, other approaches should be implemented.

Predators

The predators most commonly used in mosquito control target larvae are: fishes such as *Gambusia affinis* [50] and *Oreochromis niloticus* [51], larvae of the mosquito *Toxorhynchites* spp [52, 53] and copepods such as *Mesocyclops* and *Macrocycyclops* spp [54, 55]. The mosquitofish, *Gambusia affinis,* is tolerant to larvicides [56]; thus, this predator has the potential to be used with existing chemical approaches. They could potentially kill larvae that are resistant to insecticides.

To incorporate any predator into biological control measures, it is imperative to have an in depth knowledge of the biology and behavior of the introduced predator to avoid a negative effect on native biodiversity [57]. Thus, before introducing a non-native species, it is important to search for a native fish that can be used. Sometimes predators just need dispersal to be effective [58].

The use of microorganisms to control mosquito populations

Another approach to control mosquito populations by biological control is the introduction of microorganisms like viruses, bacteria, fungus and nematodes into a system.

Bacillus thuringensis israelensis (*Bti*) is a gram-positive bacterium. Worldwide, it is the main biological agent for controlling mosquito populations. It is a midgut "poison" for mosquito larvae. It must be ingested to be effective. Larvae ingest sporulated cells which contain the δ-endotoxins. Once in the midgut, the endotoxin dissolves due to alkaline conditions, then the endotoxin is activated by proteolytic enzymes. Toxic crystals bind to the microvillar membrane to form many small pores in the midgut epithelial cells, causing osmotic unbalance. The midgut epithelium cells swell, lyse and eventually the whole midgut is damaged, leading to generalized paralysis of the insects [23, 59]. *Bti* has a different mode of action, making it a good alternative for populations that are resistant to many chemical; for example, there is no cross-resistance with temephos-resistant populations [60].

Entomopathogenic fungus.- Fungal diseases are common in mosquitoes and can diminish their survival. Fungi from the genera *Coelomomyces*, *Beauveria*, *Lagenidium* and *Metarhizium* are the most studied and all are known to infect mosquito populations. *Coelomomyces* and *Lagenidium* have shown larvicidal activity in *Anopheles* and *Aedes* mosquitoes [61, 62] while *Beauveria* and *Metarhizium* have shown adulticidal activity against mosquitoes, decreasing the likelihood of blood feeding, survival and fecundity [63]. *Metarhizium* spp have been evaluated on impregnated cloths to control *A. aegypti* in combination with imidacloprid [64, 65].

Insecticide resistance evolution

The Insecticide Resistance Action Committee (IRAC) defines insecticide resistance as the "heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species". **Figure 1.5** illustrates the evolution of insecticide

resistance in a mosquito population over generations. In nature, there are mosquitos that have a genetic predisposition to survive insecticide exposure. Such predispositions initially occur at very low frequency (**Fig 1.5A**). Exposure to insecticides kills susceptible individuals; therefore, those mosquitoes that are resistant to the insecticide will survive along with some susceptible mosquitoes that were in a places where the insecticide could not reach them (**Fig 1.5B**). Mosquitos will then mate and reproduce and these mosquitoes will pass their genetic information to the next generation (**Fig 1.5C**). The continued application of the same insecticide kills the susceptible mosquitoes, but leaves behind resistant individuals, producing more resistant individuals (**Fig 1.5D**), until the population is mostly resistant to a particular insecticide (**Fig 1.5E**).

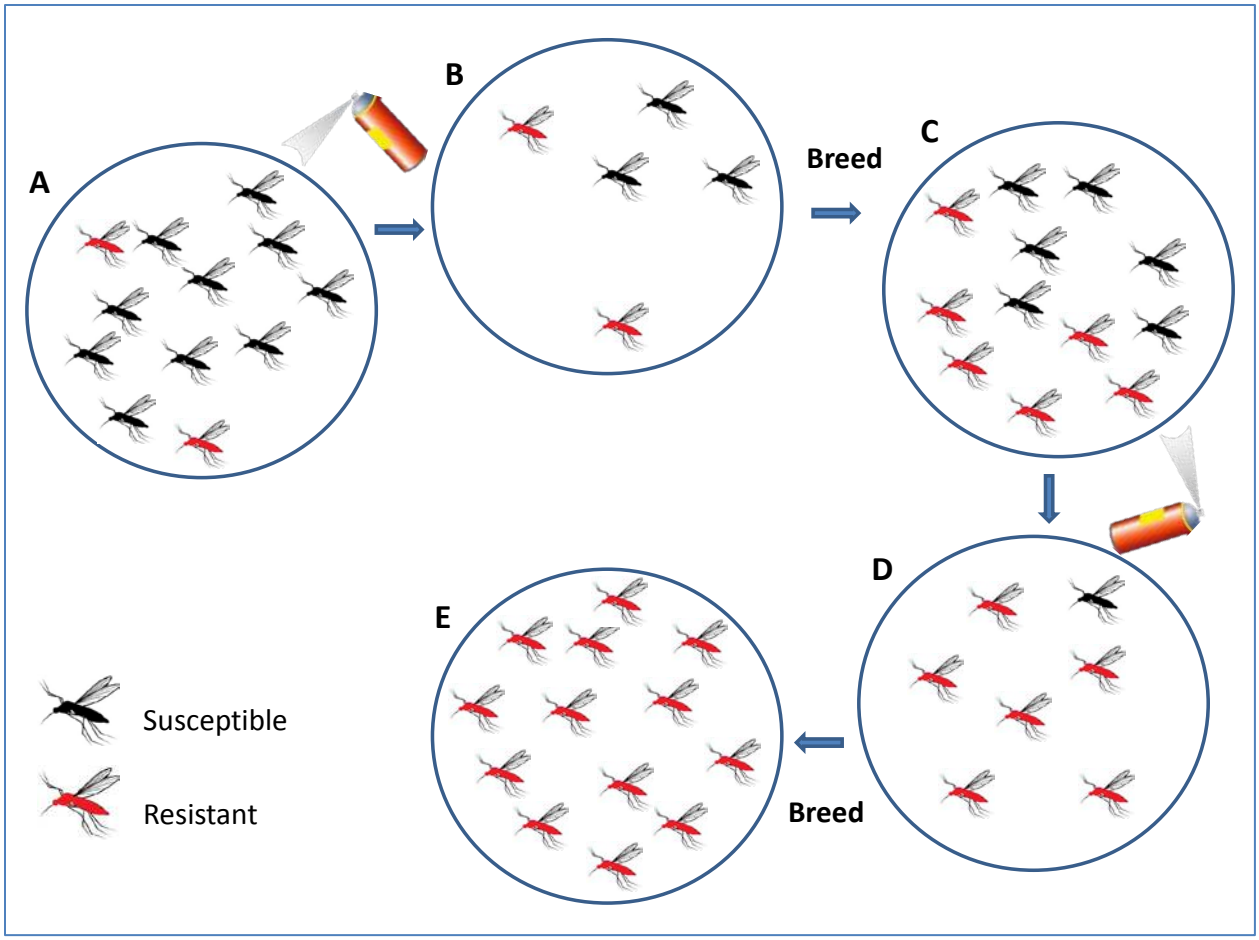


Figure 1.5. Evolution of insecticide resistance in mosquito populations over generations. In nature, some mosquitos have a genetic predisposition to survive insecticide exposure. (Fig 1.5A). Exposure to insecticides kills susceptible individuals; (Fig 1.5B). Mosquitos will then mate and reproduce and these mosquitoes will pass their genetic information to the next generation (Fig 1.5C). The continued application of the same insecticide kills the susceptible mosquitoes, but leaves behind resistant individuals, producing more resistant individuals (Fig 1.5D), until the population is mostly resistant to a particular insecticide (Fig 1.5E).

Point mutations resistance

Insects have developed different resistance mechanisms against insecticides, and there are four main mechanisms of resistance: metabolic resistance, altered target site resistance, behavioral resistance, and permeability resistance. A combination of resistance mechanisms may play a role in the resistance for a given insecticide, but the relative importance of each mechanism varies in each population and also depends on the insecticide´s mode of action. Insecticides primarily target the nervous system. An alteration in the binding site of the

insecticide can disrupt the efficacy of the chemical. Voltage gated sodium channels (vgsc), Acetycholinesterase (ACE) and GABA receptors are the three main target sites of insecticides; and therefore, mutations in these sites have been found in insecticide resistant individuals. A major mechanism of pyrethroid resistance in *Ae.aegypti* is point mutations in the vgsc.

DDT and Pyrethroids target the vgsc. Mutations in the vgsc disrupt the function of the insecticide. *Ae. aegypt*i have been found to have eleven mutations in pyrethroid resistant populations (**Table 1.2**). Eighty-one percent of the mutations (9) are located in domain II and III, and most of them are located in segment six (**Figure 1.6**), in the activation gate of the vgsc. Interestingly, various mutations seem to be region specific. The I1,016 mutation is widespread in the Americas but has not been found elsewhere. The G1,016 mutation is widespread in pyrethroid resistant Asian populations. For more detailed review of mutations in pyrethroid resistant *Ae. aegypti* populations please refer to Du et al (2016).

Table 1.2. Mutations found to date in pyrethroid resistant *Aedes aegypti* populations.

Locus	Amino acid in Susceptible	Amino acid in Resistance	Functionality confirmed	Reference
419	Valine	Leucine	Yes	Haddi et al., 2017
923	Glycine	Valine		Brengues et al., 2003
982	Leucine	Tryptophan		Brengues et al., 2003
989	Serine	Proline	Yes	Srisawat et al., 2010
1011	Isoleucine	Methionine/ Valine	Yes (M)	Brengues et al., 2003 Saavedra-Rodriguez et al., 2007
1016	Valine	Glycine/ Isoleucine	Yes (G)	Brengues et al., 2003 Saavedra-Rodriguez et al., 2007
1520	Threonine	Isoleucine		Kushwah et al., 2015
1534	Phenylalanine	Cysteine	Yes	Yanola et al., 2009
1763	Aspartic Acid	Tyrosine		Chang et al., 2009

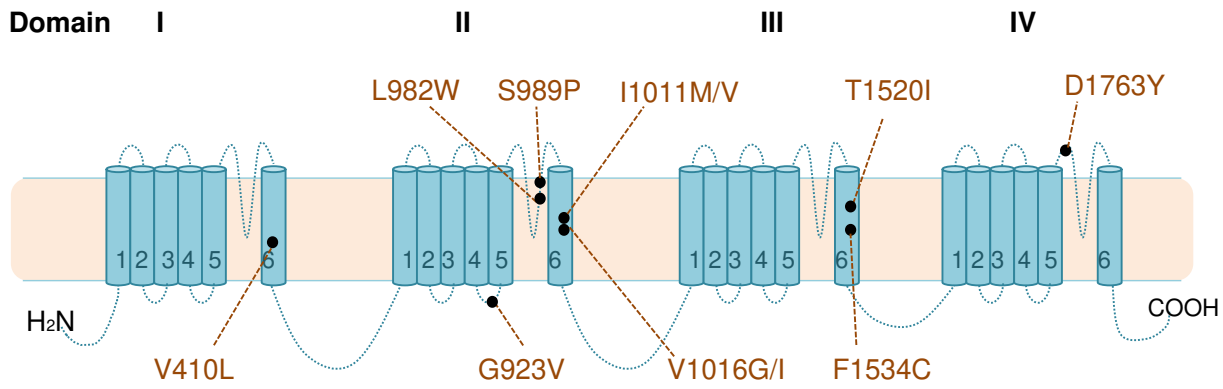


Figure 1.6. Pyrethroid resistance-associated mutations identified in the voltage-gated sodium channel of *Aedes aegypti*.

Chapter 2: Coevolution of the I1,016 and C1,534 mutations in the voltage gated sodium channel gene of *Aedes aegypti* in Mexico

Introduction

Worldwide, *Aedes aegypti* (L.) mosquitoes are the principal urban vectors of dengue, chikungunya, and yellow fever viruses. Approximately 2.5 billion people (40% of the human population) currently live with the risk of dengue transmission. In Mexico, *Ae. aegypti* is the primary vector of the four dengue virus serotypes (DENV1-4), the causative agents of dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Mexico is severely affected by DF, DSS, and DHF because all four dengue serotypes co-occur in most states of Mexico. A recent review of dengue disease in Mexico [66] reported an increase in incidences from 1.72 per 100,000, in 2000, to 14.12 per 100,000, in 2011.

Currently, the most effective means to reduce dengue transmission by *Ae. aegypti* is through reduction of larval and adult populations. In Mexico, larval reduction is accomplished chiefly through the application of the organophosphate, temephos, to peridomestic larval breeding sites and through physical source reduction or alteration of potential water-holding containers. Following recommendations of the official Mexican policy for vector control, (NOM-032-SSA2- 2002), pyrethroids were almost exclusively used to control adults in and around homes from 1999 to2010.

Pyrethroid insecticides prolong the opening of the voltage gated sodium channel protein (vgsc) in insect nerves to produce instant paralysis and ‘‘knock-down.” The α-subunit of vgsc has four repeat domains, labeled I-IV, each of which contains six transmembrane helix segments, S1-S6. Pyrethroids preferentially bind to the open state of vgsc by interacting with two distinct receptor sites formed by the interfaces of the

transmembrane helix S6 of domains II and III, respectively [29]. Early computer modeling studies [44] suggest that simultaneous binding of pyrethroids to S6 in both domains II and III is necessary to efficiently lock sodium channels in the open state. These models also predict that mutations in the S6 of domain III allosterically alter S6 in domain II via a small shift of IIS6, thus establishing a molecular basis for the coevolution of S6 mutations in domains II and III in conditioning pyrethroid resistance.

In 2006 we described a mutation, I 1,016, in the S6 of domain II in *Ae. aegypti* that is associated with very high knock-down resistance (*kdr*) to the pyrethroid insecticide permethrin in mosquitoes homozygous for this mutation. We examined collections of *Ae. aegypti* from Mexico during 1996−2009 [67] and found that the overall I1,016 frequency increased from 0.1% in 1996−2000, to 2%−5% in 2003−2006, to 38.3%−88.3% in 2007−2009 depending upon collection location. In 2010 another vgsc mutation was described in the S6 of domain III in *Ae. aegypti* that was also strongly correlated with *kdr* and involved a cysteine replacement (C1,534Phe) [68-70]. A general trend in these studies was that C1,534 frequencies were generally higher and increased more rapidly than I1,016 frequencies in natural populations.

Based upon these observations and on the dual binding model [44], we analyzed newly collected DNA from *Ae. aegypti* for I1,016 and C1,534 while DNA previously analyzed for I1,016 [67] were tested for the presence of C1,534. The purpose of this study was to test the hypothesis that mutations in the S6 of domains II and III coevolve in a dependent manner through various allosteric interactions as suggested by computer models [44, 71]. An analysis of linkage disequilibrium was performed on the two alleles in 1,016 (V1,016 (susceptible), I1,016(resistant)) and on the two alleles in 1,534 (F1,534 (susceptible), C1,534

(resistant)) to assess whether alleles at 1,534 and 1,016 evolve independently or in a correlated fashion through epistasis.

Methods

Mosquito collections

Larval mosquitoes were collected from the locations mapped in **Figure 2.1** and listed in **Table S2.1**. At each collection site, we collected immature stages from at least 30 different containers in each of three different areas located at least 100 m apart. This included water storage containers and discarded trash containers such as plastic pails, tires, and cans. Larvae were returned to the laboratory where they were reared to adults and then identified to species. The Viva Caucel collection was west of the city of Merida in Yucatán State (20.9979639°, 089.7174611°). The Vergel collection was from eastern Merida (**Figure 2.1**) (20.9575694°, -89.5886889°). Both were collected in 2011 by Universidad Autónoma de Yucatán. DNA was isolated from individual adult mosquitoes by the salt extraction method [72] and suspended in 150 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The SNP identification, allele-specific polymerase chain reaction (PCR), melting curve conditions, and genotype readings followed published procedures [69, 73-75].

Figure 2. 1. Location of samples sites of *Ae. aegyti* from Mexico.

Association between *vgsc* genotypes and kdr phenotypes

The F_3 generation of the Viva Caucel and Vergel strains were exposed to 25 μg permethrin (Chem Service, West Chester, PA) coated 250 mL Wheaton bottles. In each bottle approximately fifty 3–4 days old mosquitoes were exposed for one hour. Active mosquitoes were transferred to cardboard cups and frozen at -80°C and formed the 'alive' group. Knocked down mosquitoes were transferred to a second cardboard cup and placed into an incubator at 28°C and 70% humidity. After four hours, newly recovered mosquitoes were aspirated, frozen and labeled as 'recovered'. The mosquitoes that remained inactive were scored as 'dead'.

Linkage disequilibrium analysis

There are four potential 1,016/1,534 di-locus haplotypes: V1,016/F1,534 (VF), V1,016/C1,534 (VC), I1,016/F1,534 (IF), I1,016/C1,534 (IC). The number of times (T_{ij}) that an allele at locus i = 1,016 appears with an allele at locus j = 1,534 was determined by the program LINKDIS [76]. The program then calculated composite disequilibrium frequencies [77] because the phase of alleles at 1,016 and 1,534 are unknown in double heterozygotes. An unbiased estimate of the composite disequilibrium coefficient Δ_{ij} [77, 78] was calculated as:

$$\Delta_{ij} = \left(\frac{N}{N-1}\right)\left(\left(\frac{T_{ij}}{N}\right) - 2P_iP_j\right)$$

Where N is the sample size and P_i and P_j are the frequencies of alleles at locus i = 1,016 and locus j = 1,534 respectively. Bayesian 95% Highest Density Intervals (HDI) around p_i and P_j were calculated in WinBUGS [79].

A χ^2 test was performed to determine if significant disequilibrium exists among all alleles at 1,016 and 1,534. The statistic was calculated and summed over all two-allele-interactions [78]:

$$X^2_{[1\ d.f.]} = N \sum_i \sum_j \left(\frac{\Delta^2_{ij}}{P_i P_j}\right)$$

The linkage disequilibrium correlation coefficient R_{ij} [78] is distributed from -1 (both mutations *trans*) to 0 (1,534 and 1,016 mutations occur independently), to 1 (both mutations *cis*) and therefore provides a standardized measure of disequilibrium:

$$R_{ij} = \frac{\Delta_{ij}}{\sqrt{P_i(1-P_i) + C_i)(\ P_j\ (1-P_j) + C_j\ )}}$$

Where the C_i term corrects for departures from Hardy-Weinberg expectations:

$$C_i = H_{obs}(i) - P_i^2$$

where $H_{obs}\ (i)$ is the observed frequency of i homozygotes. Departures from Hardy-Weinberg expectations were also expressed as Wright's inbreeding coefficient (F_{IS}) and calculated as 1- ($H_{exp}/2p\ (1-\ p)$) where H_{exp} is the observed frequency of heterozygotes. A χ^2 test of the hypothesis $F_{IS} = 0$ with one degree of freedom is:

$$X^2_{[1\ d.f.]} = \frac{N\left(H_{exp} - H_{obs}\right)^2}{\sum_i P_i^2 + (\sum_i P_i^2)^2 - 2\sum_i P_i^3}$$

Results

Associations between *vgsc* genotypes and kdr phenotypes

The locations of all sampling sites are shown in **Figure 2.1** and the latitude and longitude coordinates are provided in **Table S2.1.** The sample sizes and numbers of the nine di-locus genotypes (Three 1,534 genotypes x Three 1,016 genotypes) are listed in **Table S2.1.** From a total of 615 treated mosquitoes in Viva Caucel, 17.6% (n = 108) were scored as alive, 15.6% (n = 96) as recovered and 66.8% (n = 411) as dead. Genotypes at 1,016 and 1,534 were identified in 95 randomly chosen individuals from each group (**Table 2.1**). From a total of 337 treated Vergel mosquitoes, 48.1% (n = 162) were scored as alive, 20.5% (n = 68) as recovered and 31.5% (n = 106) as dead. We randomly chose 95, 66 and 96 Vergel individuals from each group, respectively to obtain the genotypes at 1,016 and 1,534 (**Table 2.1**).

In Viva Caucel, the frequency of the I1,106 allele was 0.746 and the frequency of the C1,534 allele was 0.926 (**Table 2.2**). In Vergel I1,016 was at a slightly higher frequency of 0.80 while the C1,534 allele was close to fixation at 0.988. The I1,106 and C1,534 alleles were in positive disequilibrium in Viva Caucel, but were only marginally significant in Vergel.

Table 2. 1. Phenotypes and genotypes at loci 1,016 and 1,534 in Viva Caucel and Vergel.

Site	Genotype		Phenotype			Total
	V1,016I	F1,534C	Alive	Recovered	Dead	
Viva Caucel						
	II	CC	91	66	11	168
	II	CF	0	0	0	0
	II	FF	0	0	0	0
	IV	CC	0	28	40	68
	IV	CF	3	0	17	20
	IV	FF	0	0	0	0
	VV	CC	0	1	7	8
	VV	CF	0	0	18	18
	VV	FF	1	0	1	2
	Total		95	95	94	284
Vergel						
	II	CC	87	43	26	156
	II	CF	1	0	0	1
	II	FF	0	0	0	0
	IV	CC	6	22	68	96
	IV	CF	0	1	0	1
	IV	FF	0	0	0	0
	VV	CC	0	0	1	1
	VV	CF	0	0	0	0
	VV	FF	1	0	1	2
	Total		95	66	96	257

Table 2. 2. Genotype and allele frequencies at loci 1,016 and 1,534 in Viva Caucel and Vergel.

Locus	Genotype	Viva Caucel	Vergel
1,016			
	II	168	157
	VI	88	97
	VV	28	7
	Allele frequency I	0.746	0.8
	Allele frequency V	0.254	0.2
1,534			
	CC	244	253
	FC	38	2
	FF	2	2
	Allele frequency C	0.926	0.988
	Allele frequency F	0.074	0.012

Genotypes at the 1,016 and 1,534 loci were not independent, in agreement with the linkage disequilibrium analysis in **Table 2.3**. **Table 2.4** is a three-way contingency analysis of genotypes at loci 1,016, 1,534 and numbers of alive or dead individuals in Viva Caucel and Vergel. Number of alive mosquitoes were not independent of genotypes at the 1,016 locus; specifically, number of alive mosquitoes were significantly greater in I1,016 homozygous mosquitoes than in heterozygotes or in V1,016 homozygotes. Number of alive mosquitoes were also not independent of genotypes at the 1,534 locus; specifically, the number of alive mosquitoes were significantly greater in C1,534 homozygous mosquitoes than in heterozygotes or in F1,534 homozygotes. In general, the number of alive mosquitoes in the Viva Caucel strain was not independent of genotypes at either locus.

Table 2. 3. Linkage disequilibrium analysis at loci 1,016 and 1,534 in Viva Caucel and Vergel.

Collection site	Di-locus Genotype	Observed	Expected	R_{ij}	χ^2	prob.
Viva Caucel						
	$I_{1,016}/F_{1,534}$	10	31.4	-0.6628	124.75	5.76E-29
	$I_{1,016}/C_{1,534}$	414	392.6	0.6628		
	$V_{1,016}/F_{1,534}$	32	10.6	0.6628		
	$V_{1,016}/C_{1,534}$	112	133.4	-0.6628		
Vergel						
	$I_{1,016}/F_{1,534}$	1.5	4.8	-0.1132	3.64	0.0565
	$I_{1,016}/C_{1,534}$	409.5	406.2	0.1132		
	$V_{1,016}/F_{1,534}$	4.5	1.2	0.1132		
	$V_{1,016}/C_{1,534}$	98.5	101.8	-0.1132		

Table 2. 4. Three-way tests of independence between numbers alive, recovered or dead and genotypes at vgsc loci 1,016 and 1,534.

Site	Hypothesis tested	G	d.f.	Prob.	Hypothesis supported?
Viva Caucel- No.alive vs dead					
	1,016 X 1,534 independent?	77.7(33%)	4	5.48E-16	No
	1,016 X No. alive independent?	139.1(60%)	2	6.35E-31	No
	No. alive = in II (54.2%) vs VI (3.4%)?	-113.8	-1	1.44E-26	No
	No. alive = in II (54.2%) vs VV (3.5%)?	-65.75	-1	5.12E-16	No
	No. alive = in VI (3.4%) vs VV (3.5%)?	-0.22	-1	6.41E-01	Yes
	1,534 x No. alive independent?	33.02(14%)	2	6.75E-08	No
	No. alive = in CC (38.6%) vs FC (7.9%)?	-4.77	-1	2.89E-02	No
	No. alive = in CC (38.6%) vs FF (1/2)?	-0.35	-1	5.53E-01	Yes
	No. alive = in FC (7.9%) vs FF (1/2)?	-33.02	-1	9.12E-09	No
	1,016 X 1,534 x No. alive interaction	-17.91(-8%)	4	-	-
	1,016 X 1,534 x No. alive independent?	231.83	12	8.32E-43	No
Vergel- No. alive versus dead					
	1,016 X 1,534 independent?	27.93(23%)	4	1.29E-05	No
	1,016 X No. alive independent?	88.27(73%)	2	6.81E-20	No
	No. alive = in II (56%) vs VI (6.2%)?	-88.27	-1	5.72E-21	No
	No. alive = in II (56%) vs VV (1/3)?	-2.83	-1	9.25E-02	No
	No. alive = in VI (6.2%) vs VV (1/3)?	-7.66	-1	5.63E-03	No
	1,534 x No. alive independent?	0.53(0%)	2	7.69E-01	Yes
	No. alive = in CC (36.8%) vs FC (1/2)?	-0.25	-1	6.14E-01	Yes
	No. alive = in CC (36.8%) vs FF (1/2)?	-0.42	-1	5.15E-01	Yes
	No. alive = in FC (1/2%) vs FF (1/2)?	0	-1	9.61E-01	Yes
	1,016 X 1,534 x No. alive interaction	4.94(4%)	4	2.94E-01	Yes
	1,016 X 1,534 x No. alive independent?	121.66	12	2.88E-20	No
Viva Caucel- Recovered versus dead					
	1,016 X 1,534 independent?	56.65(40%)	4	1.47E-11	No
	1,016 X recovery independent?	70.51(49%)	2	4.89E-16	No
	No. recovered = in II (39.3%) vs VI (31.8%)?	-45.24	-1	1.74E-11	No
	No. recovered = in II (39.3%) vs VV (3.6%)?	-53.26	-1	2.92E-13	No
	No. recovered = in VI (31.8%) vs VV (3.6%)?	-6.45	-1	1.11E-02	No
	1,534 x recovery independent?	44.20(31%)	2	2.52E-10	No
	No. recovered = in CC (39.8%) vs FC (0%)?	-5.14	-1	2.33E-02	No
	No. recovered = in CC (39.8%) vs FF (0/2)?	-0.96	-1	3.26E-01	Yes
	No. recovered = in FC (0%) vs FF (0/2)?	-44.04	-1	3.21E-11	No
	1,016 X 1,534 x recovery interaction	-27.95(-19%)	4	-	-
	1,016 X 1,534 x recovery independent?	143.4	12	1.23E-24	No
Vergel- Recovered versus dead					
	1,016 X 1,534 independent?	17.59(42%)	4	1.49E-03	No
	1,016 X recovery independent?	21.07(50%)	2	2.66E-05	No
	No. recovered = in II (27.4%) vs VI (23.7%)?	-21.01	-1	4.58E-06	No
	No. recovered = in II (27.4%) vs VV (0/3)?	-1.67	-1	1.97E-01	Yes
	No. recovered = in VI (23.7%) vs VV (0/3)?	-0.4	-1	5.27E-01	Yes
	1,534 x recovery independent?	0.71(2%)	2	6.99E-01	Yes
	No. recovered = in CC (25.7%) vs FC (1/2)?	-0.29	-1	5.92E-01	Yes
	No. recovered = in CC (25.7%) vs FF (0/2)?	-0.01	-1	9.21E-01	Yes
	No. recovered = in FC (1/2) vs FF (0/2)?	-0.71	-1	3.99E-01	Yes
	1,016 X 1,534 x recovery interaction	2.39(6%)	4	6.64E-01	Yes
	1,016 X 1,534 x mortality independent?	41.76	12	3.65E-05	No

One problem that exists with this analysis is that genotypes at the two loci are not independent. In this and previous studies [73, 74], I1,016 homozygous mosquitoes have the greatest survival, while few, if any heterozygotes or V1,016 homozygotes survive. To evaluate C1,534 genotypes independently of I1,016 homozygous mosquitoes, we only compared the three C1,534 genotypes among I1,016 heterozygotes and V1,016 homozygotes. A significantly larger proportion of C1,534 homozygotes survived.

Table 2.4 also shows the contingency analyses of Vergel mosquitoes. Genotypes at the 1,016 and 1,534 loci were not independent, while they were marginally significant in the linkage disequilibrium analysis shown in **Table 2.3**. Numbers of mosquitoes alive were not independent of genotypes at the 1,016 locus again because numbers of mosquitoes alive were significantly greater in I1,016 homozygous mosquitoes than in heterozygotes or in V1,016 homozygotes. Numbers of alive were independent of genotypes at the 1,534 locus, specifically because C1,534 was almost fixed in the Vergel strain.

Table 2.4 also shows the three-way contingency analysis between genotypes at loci 1,016 and 1,534 and the numbers of recovered or dead mosquitoes in Viva Caucel. As in **Table 2.3**, genotypes at the 1,016 and 1,534 loci were not independent. The number of recovered mosquitoes was not independent of genotype at the 1,016 locus; specifically, numbers of recovered mosquitoes were significantly greater in I1,016 homozygous mosquitoes than in heterozygotes or in V1,016 homozygotes. Numbers of recovered mosquitoes were also not independent of genotypes at the 1,534 locus; specifically, numbers of alive mosquitoes were significantly greater in C1,534 homozygous mosquitoes than in heterozygotes or in F1,534 homozygotes.

In general, numbers of recovered mosquitoes in the Viva Caucel strain were heavily dependent on genotypes at both loci. An interesting difference between the two loci is that 32% (28/88) of I1,016 heterozygotes recovered while only 3.6% (1/28) of C1,534 heterozygotes recovered. This difference was significant ($\chi^2 = 7.59$, df = 1, p-value = 0.006).

Table 2.4 also shows the same trend in recovery in Vergel mosquitoes. Genotypes at the 1,016 and 1,534 loci were not independent, while they were marginally significant in the linkage disequilibrium analysis in **Table 2.3**. Numbers of recovered mosquitoes were not independent of genotypes at the 1,016 locus, again because numbers of recovered mosquitoes were significantly greater in I1,016 homozygous mosquitoes than in heterozygotes or in V1,016 homozygotes. However, numbers of recovered mosquitoes were independent of genotypes at the 1,534 locus, specifically because C1,534 was approaching fixation in the Vergel strain.

Spatial and temporal analysis of genotype frequencies

Table S2.2 contains the frequencies of I1,016 and C1,534 and their Bayesian 95% HDI. F_{IS} was significantly greater than zero (heterozygote deficiency) in two of the 36 collections where I1,016 and V1,016 alleles were segregating. In contrast, a significant heterozygote deficiency occurred in eight of the 53 collections where C1,534 and F1,534 were segregating and a heterozygote excess occurred in two collections.

The frequencies of the I1,016 and C1,534 alleles from 1999 to 2012 are plotted in **Figure 2.2**. The C1,534 allele appeared sooner and increased more rapidly than I1,016. Only the states of Veracruz and Chiapas had sufficient samples over the years to compare the spatial distributions of I1,016 and C1,534 (**Figure 2.3**). It is very clear that I1,016 and C1,534 were increasing in frequency much earlier in Veracruz state in eastern Mexico than in

Chiapas state in southwestern Mexico. It is also clear that in both states C1,534 was increasing in frequency much earlier than I1,016. Starting in 2002, the frequency of C1,534 was greater than or equal to that of I1,016. In a yearly comparison of *Ae. aegypti* collection sites, 80 out of 87 sites (**Table S2.2**) had a frequency of C1,534 greater than the frequency of I1,016. In 6 of the 7 cases where the frequency of I1,016 exceeded that of C1,534, the difference was only from 1–2% and values were not significant different (overlapping 95% HDI). Only in Martınez de la Torre in 2002 was there a significant difference of 9%.

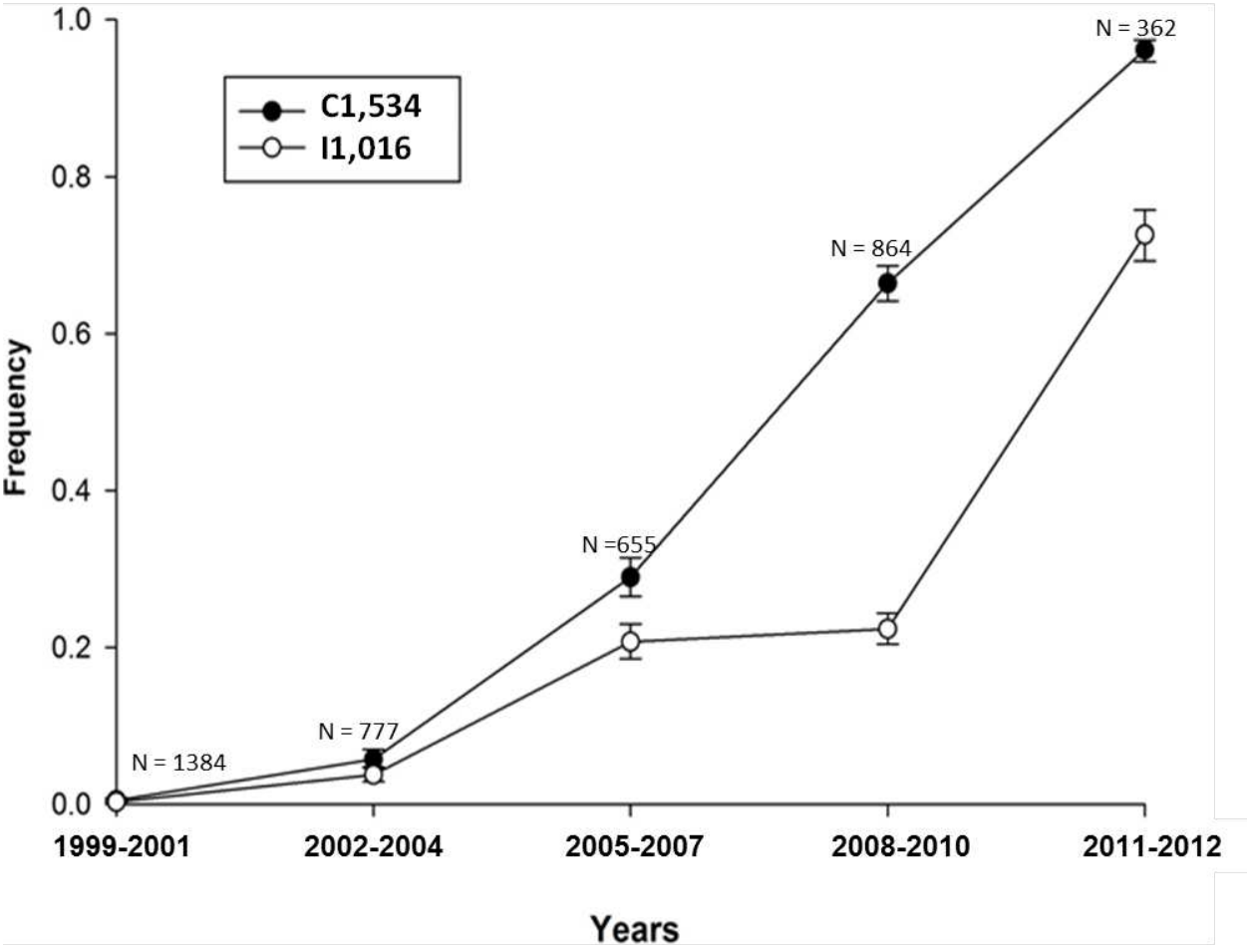


Figure 2. 2. Frequencies of I1,016 and C1,534 alleles from 1999 and their Bayesian 95% HDI.

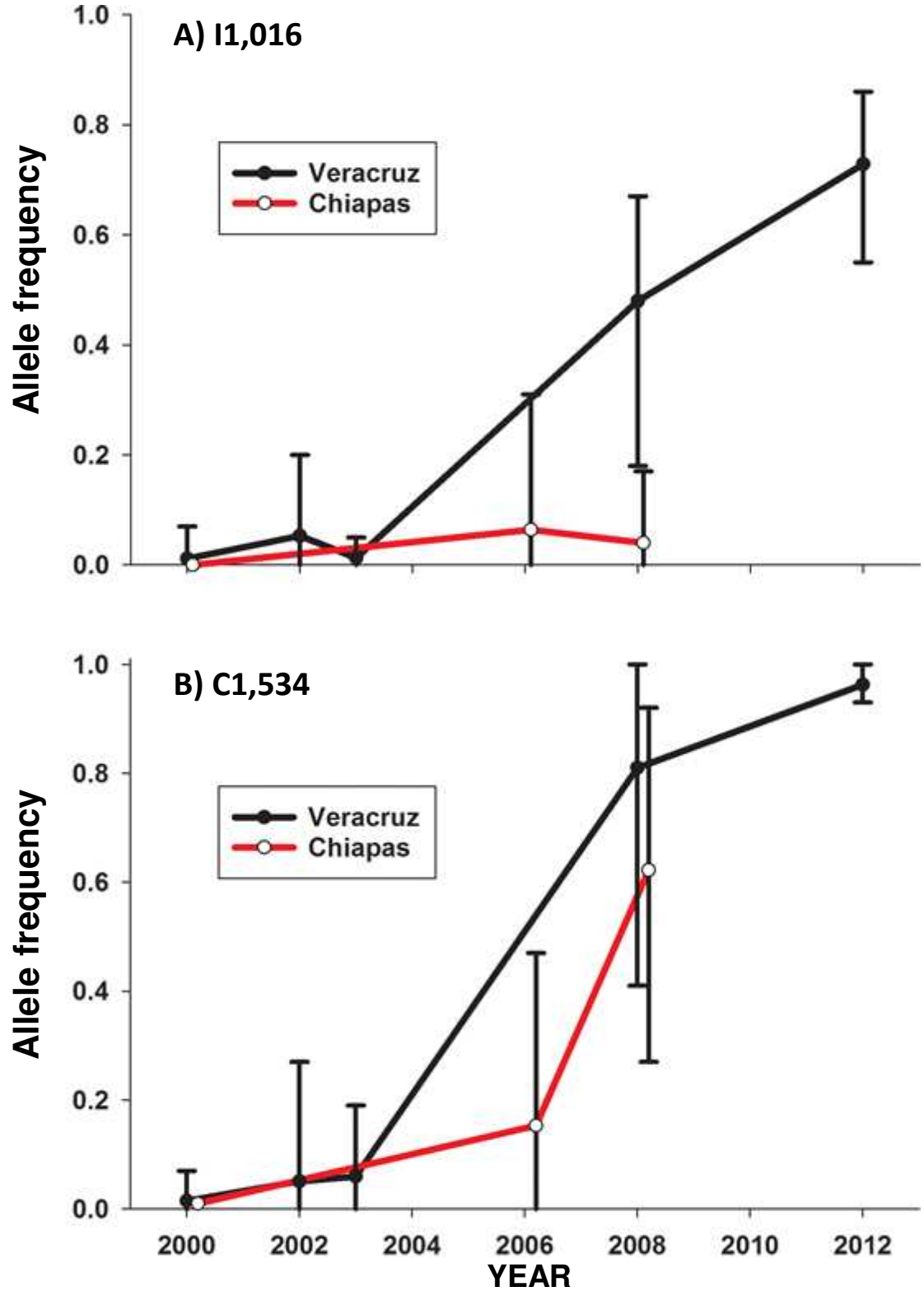


Figure 2. 3. Frequencies of I1,016 (A) and C1,534 (B) alleles from 2000 to 2012 in the cities of Veracruz and Chiapas and their maximum and minimum frequencies among collections in each year.

Linkage disequilibrium analysis

Linkage disequilibrium analysis can only be performed in datasets where alleles are segregating at both loci. There were 34 datasets that met this criteria of the 87 collections listed in **Table S2.1**. **Table S2.3** lists the state, city and year of the 34 datasets along with linkage disequilibrium correlation coefficient Rij and its associated $\chi 2$ values and the probability of a greater $\chi 2$. I1,016 and C1,534 were in disequilibrium in the majority (21/34 = 62%) of datasets. For the most part, alleles in 1,534 and 1,016 were evolving in a correlated, dependent fashion. However, this analysis does not provide specific information about the four haplotypes.

The frequencies of the four potential di-locus haplotypes are plotted by year in **Figure 2.4**. The frequency of the susceptible V1,016/F1,534 (VF) haplotype remained high from 1999–2003 (**Figure 2.4A**). No collections were made again until 2008, by which time frequencies had dropped to 0–0.6. Four years later, VF was approaching extinction in all collections. **Figure 2.4B** plots the frequency of the V1,016/C1,534 (VC) haplotype. From 1999–2003, VC frequencies remained low (0–0.10). By 2008, frequencies had increased to 0.1–0.75. Four years later, VC was declining in frequency in two collections and was increasing in four collections. A very different trajectory occurred for I1,016/F1,534 (IF) (**Figure 2.4C**). From 1999–2002, the IF frequency remained low and only reached as high as 0.1 in two collections. By 2008 frequencies were approaching extinction and four years later similar trends were seen, even though VC and IC frequencies had increased dramatically. **Figure 2.4D** is a plot of the frequency of the resistant I1,016/C1,534 (IC) haplotype. From 1999–2002, the IC frequency was low and only reached 0.1 in one collection. By 2008

frequencies had increased dramatically in all collections and continued to increase in all collections up to 2012 when frequencies ranged from 0.5−0.9.

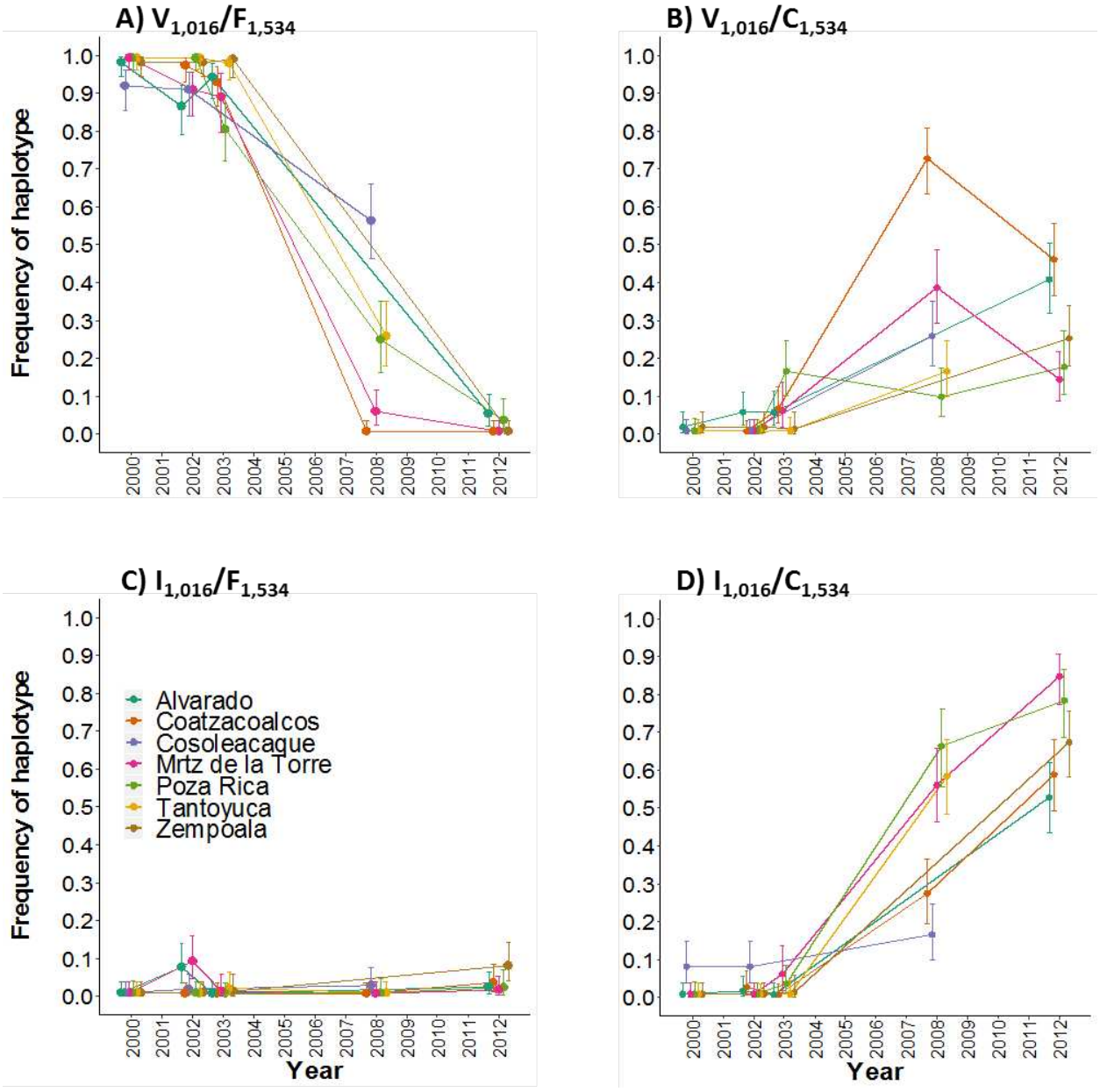


Figure 2. 4. Frequencies of the four potential di-locus haplotypes plotted by year. A) Frequency of the susceptible V1,016/F1,534(VF) haplotype, B) Frequency of the V1,016/C1,534 (VC) haplotype, C) Frequency of the I1,016/F1,534 haplotype and D) Frequency of the resistant I1,016/C1,534 (IC) haplotype.

Discussion

The frequency of C1,534 has increased dramatically in the last decade in several states in Mexico including Nuevo Leon in the north, Veracruz on the central Atlantic Coast, and Chiapas, Quintana Roo and Yucatan in the south. The linkage disequilibrium analysis on the I1,016 and C1,534 alleles in *Ae. aegypti* collected in Mexico from 2000–2012 (**Table 2.3**) strongly supports statistical associations between 1,534 and 1,016 mutations in natural populations. Furthermore, the dynamics of haplotype frequencies during that time suggest pyrethroid resistance in the *vgsc* gene requires the sequential evolution of 1,534 and 1,016 mutations. **Figure 2.4C** suggests that the I1,016/F1,534 haplotype has a low fitness, even when pyrethroids are being released. For this reason I1,016 is unlikely to have evolved independently. Instead it is much more likely that the C1,534 mutation evolved first but conferred only a low level of resistance. This conjecture is strongly supported by the fact that in 80 of 87 collections (92%), the frequency of C1,534 was greater than the frequency of I1,016.

The findings of this study are different in many respects from those in a study of a Y1,575 substitution in *Anopheles gambiae* that occurs just beyond the S6 of domain III, within the linker between domains III and IV [80]. This linker contains a sequence of three amino acids (IFM) that close the sodium channel pore following activation, which blocks the influx of sodium into the cell and restore the membrane resting potential. In contrast, C1,534 in *Ae. aegypti* occurs in the S6 of domain III. This is close to a M1,524I substitution that has been associated with knockdown resistance in *Drosophila melanogaster* [81] and a F1,538I mutation that reduces sensitivity to deltamethrin in arthropods and mammals [82, 83].

Mutations in S6 of domain II, such as F1,014, S1,014 in *An. gambie* and I1,016 and G1,016 in *Ae. aegypti* are not directly in the binding pocket, but affect the resistance phenotype by preventing binding of insecticides and changing the conformation of the vgsc [44, 84]. In contrast, a binding site located in a hydrophobic cavity delimited by the IIS4-S5 linker and the IIS5/IIIS6 helices has recently been proposed [85] that is accessible to the lipid bilayer and lipid-soluble insecticides. The methylcyclopropane (or equivalent structure) of pyrethroids and the trichloromethyl group of DDT appear to be critical features for the action of both pyrethroids and DDT. Both insecticides fit into a slot in a small pocket in the main hydrophobic cavity, flanked by V1,529 and F1,530 on IIIS6. The binding site is formed upon opening of the sodium channel and is consistent with observations that pyrethroids bind preferentially to open channels. This binding pocket includes several known mutations in the S6 of domain III that reduce sensitivity to pyrethroids. Two nearby residues (G1,535 and F1,538) have been previously implicated in resistance in other insect species (23).

A study in which *An. gambiae* mosquitos were collected from a range of approximately 2000 km throughout West/Central Africa and had Y1,575 occurring at frequencies up to 30% in both M and S forms. Even though this mutation is seen over a large range of the continent, only a single Y1,575 haplotype occurred with a F1,014 haplotype background (possibly analogous in function to I1,016), which infers strong positive selection acting on a recent mutant [80]. In contrast to the present study, F1,014 is almost fixed in West Africa and the Y1,575 allele is increasing in frequency in M form but not in S form. Thus in contrast to the apparent evolution of I1,016 on a C1,534 background as reported here in *An. gambiae*, Y1,575 appears to have evolved on a F1,014 background.

There are many potential reasons for this difference including the possibility that mutations within the S6 of domain III may produce a different resistance mechanism and have a different impact on fitness than mutations in the linker between domains III and IV. It is also possible that the specific changes of amino acids at these sites are unique and may confer different resistance phenotypes. In either case it seems likely that one of the mutations compensates for deleterious fitness effects of the other mutation and/or confers additional resistance to insecticides.

An interesting difference between the two mutations in the present study is that 32% of I1,016 heterozygotes recover from pyrethroid exposure but only 3.6% of C1,534 heterozygotes recover. Thus while C1,534 in synergy with I1,016 may confer greater survival following pyrethroid exposure, I1,016 may confer a greater ability to recover following knockdown in heterozygotes.

There was evidence of heterozygote deficiency in eight of the 53 collections and the average F_{IS} among these eight collections was large and positive (0.580) while the average among all collections was 0.052. This suggests that the fitness of F1,534 and C1,534 homozygotes may be greater than the fitness of F1,534C heterozygotes (i.e. underdominance). While these parameters have been estimated at the 1,016 locus [86], no similar studies have involved the 1,534 locus and so the stability point beyond which the frequency of either allele would increase has not been determined. Since the C1,534 confers some degree of pyrethroid resistance (**Table 2.1-2.4**), directional selection could increase the frequency of C1,534 beyond the underdominance stability point, at which stage the frequency of C1,534 would rapidly increase towards fixation.

Little is known of other mutations in the *Ae. aegypti vgsc* that may affect pyrethroid resistance. Codon 989 in the "super-kdr" region of domain II was assessed and no mutations were found [74]. Isoleucine, M and V alleles occur at codon 1,011 [74] but these alleles were not associated with resistance in our initial survey of 1,318 mosquitoes from the 32 strains throughout Latin America [74]. The recombination dynamics of the *Ae. aegypti vgsc* are also poorly understood. Analysis of segregation between alleles at the 1,011 and 1,016 codons in generation F_3 showed a high rate of recombination even though the two codons are only separated by an approximately 250 bp intron [74]. A maximum parsimony phylogeny of the intron spanning exons 20 and 21 in 88 mosquitoes with different genotypes in exons 1,011 and 1,016 indicated the presence of three clades with bootstrap support >80%. These were arbitrarily labelled clades 1–3. The frequencies of I1,011, M1,011, V1,011, V1,016, I1,016 and G1,016 (from Thailand only) were then compared among the three clades. The frequency of I1,011 was distributed independently among the three clades, as was V1,011 and M1,011. However, there was a very evident excess of V1,016 alleles in clade 1 and an excess of I1,016 alleles in clade 2. I1,016 alleles occurred in disequilibrium with a large number of segregating sites in the intron and a large excess of I1,016 alleles were found to be associated with clade 2 in the phylogenetic analysis. This pattern is consistent with a hypothesis that a genetic sweep of the I1,016 allele and proximate intron sequences has occurred through DDT exposure and subsequently pyrethroid selection. Furthermore, the genetic sweep was recent enough that there has been insufficient time for recombination to disrupt the disequilibrium between the I1,016 allele and proximate intron sequences.

Recent work on the dual binding model may shed some light on the next steps in the evolution of pyrethroid resistance in the *vgsc* [71]. The Y1,575 mutation in *An. gambiae* was

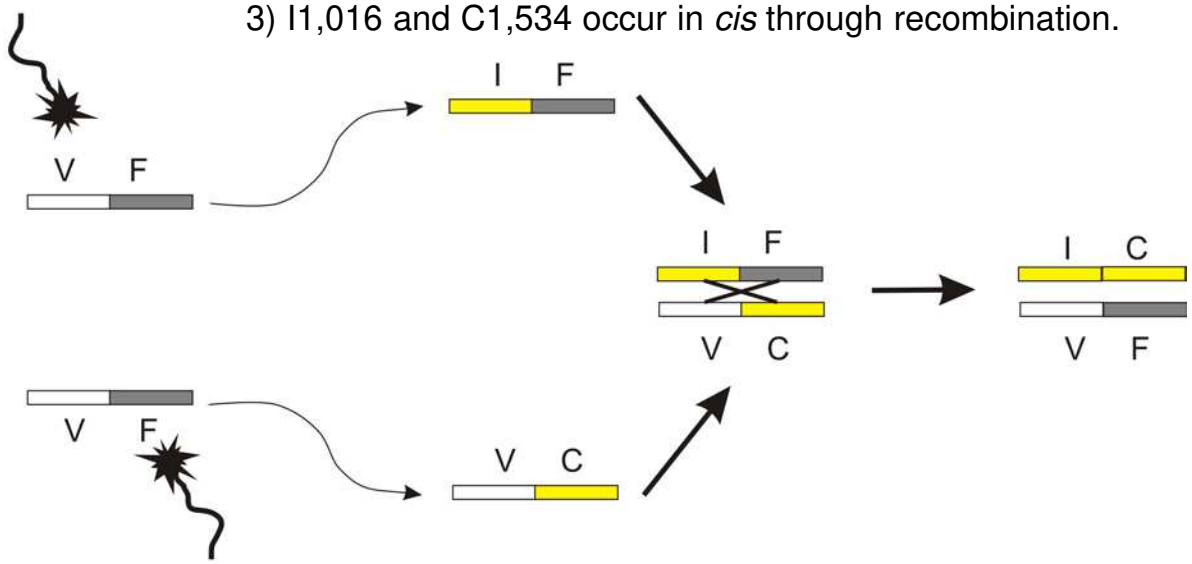
introduced alone into an *Ae.aegypti* sodium channel (AaNav1-1) [71] and then in combination with F1,014. Both substitutions were then functionally examined in *Xenopus* oocytes [71]. Y1,575 alone did not alter AaNav1-1 sensitivity to pyrethroids. However, the Y1575-F1014 double mutant was more resistant to pyrethroids than the F1014 mutant channel alone. Further mutational analysis showed that Y1,575 could also synergize the effect of S1,014 and W1,014, but not G1,014, or other pyrethroid-resistant mutations in subunit 6 of domains I or II. Computer modeling predicted that Y1,575 allosterically alters pyrethroid binding via a small shift of the subunit 6 of domain II. This establishes a molecular basis for the coexistence of Y1,575 with F1,014 in pyrethroid resistance, and suggests an allosteric inter- action between IIS6 and Loop III/IV in the sodium channel.

The rapid increase in C1,534 (**Figure 2.4B and 4D**) cannot be the result of neutral forces such as genetic drift or founder's effects. Parallel increases in C1,534 frequency occurred through- out Mexico. Even though the forces that caused an increase in the frequency of C1,534 are unclear, our results suggest that I1,016 in domain IIS6 arose from a V1,016/C1,534 haplotype and was rapidly selected possibly because double mutants confer higher pyrethroid resistance. When combined with F1014, the Y1,575 mutation in *An. gambiae* increased resistance to permethrin and deltamethrin by 9.8- and 3.4-fold, respectively [71].

Figure 2.5 illustrates two models for the evolution of 1,534 and 1,016 mutations. Model 1 proposes that the 1,534 and 1,016 mutations occurred independently and became *cis* by crossing over. Model 2 instead proposes that 1,534 mutations occurred first because 1,016 mutations confer low fitness. I1,016 mutations then arose on a V1,016/C1,534 background. These results suggest that knowledge of the frequencies of both 1,534 and 1,016

mutations are important to predict the potential of a population to evolve *kdr*. Obviously, the frequency of I1,016 by itself is a poor predictor (**Figure 2.4C**). Populations that are pyrethroid susceptible, but have high V1,016/C1,534 frequencies, are at high risk for rapid *kdr* evolution. If our experience in tracking the frequencies of I 1,016 and C1,534 mutations over the past 15 years can be extended to other *Ae. aegypti* populations, then populations with intermediate to high frequencies of C1,534 might only be susceptible for 5−10 years. Conversely, pyrethroid susceptible populations without either mutation are unlikely to develop *kdr* quickly and might be susceptible for up to 10−15 years.

Model 1: 1) V1,016 mutates to I1,016 in one haplotype.
2) Independently F1,534 mutates to C1,534.
3) I1,016 and C1,534 occur in *cis* through recombination.



Model 2: 1) V1,016 mutates to I1,016 in one haplotype but has low fitness.
2) F1,534 mutates to C1,534 that confers partial resistance.
3) V1,016 mutates to I1,016 on a C1,534 haplotype.
4) I1,016 and C1,534 occur in *cis* through sequential mutations.

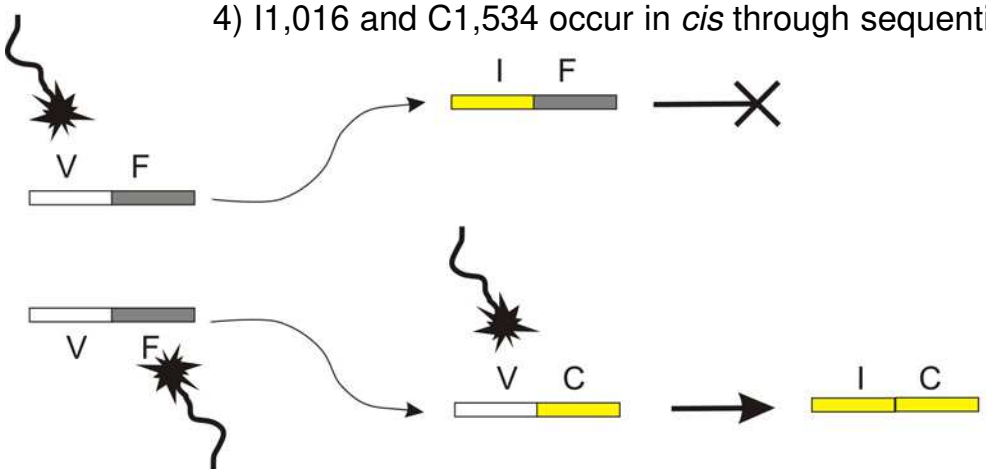


Figure 2. 5. Two models for the evolution of mutation in subunit 6 of domains II and III. Model 1 proposes that the 1,016 and 1,534 mutations occurred independently and became cis through crossing over. Model 2 instead proposes that 1,534 mutations occur first because 1,016 mutations confer low fitness. I1,016 mutations then arise on a V1,016/C1,534 background.

Chapter 3: Parallel evolution of vgsc mutations at domains IS6, IIS6 and IIIS6 in pyrethroid resistant *Aedes aegypti* from Mexico

Introduction

Pyrethroids are the most common class of insecticides used to suppress adult populations of *Aedes aegypti*, the principal vector of dengue, chikungunya, Zika and yellow fever viruses. The lack of vaccines for most of these arboviruses results in a tremendous reliance on chemical control. Unfortunately, intense application of pyrethroids has resulted in pyrethroid resistance in *Ae. aegypti* populations worldwide [68, 87-90]. A major mechanism underlying pyrethroid resistance is known as knockdown resistance (kdr), which is caused by mutations in the voltage-gated sodium channel (*vgsc*) [91]. Knowledge of the specific *vgsc* mutations that confer resistance is essential to predict the rise of pyrethroid resistance and to develop strategies for resistance management.

Globally, eleven *vgsc* mutations have been identified in *Ae. aegypti* and in most cases, have been linked to conferring some degree of pyrethroid resistance. These include G923V, L982W, I1,011 M and V1,016 G first identified in 2003 [92], I1,011 V and V1,016I in 2007 [74], D1,763Y in 2009 [93], S989P and F1,534C in 2010 [69, 94], T1,520I in 2015 [95] and V410L in 2017 [96]. These kdr mutations are usually confined to specific geographic areas and co-occurrence of certain mutations is a common phenomenon sometimes associated with higher levels of phenotypic resistance [29]. To date, only five mutations have been functionally confirmed to reduce *vgsc* sensitivity to pyrethroids, including S989P (IIL5–6), I1,011 M, V1,016 G (IIS6), F1,534C (IIIS6) [29] and most recently V410L (IS6) [96]. Computer modeling predicts that pyrethroids bind to two homologous lipid exposed interfaces between domains: one binding site (PYR-1) is formed by the linker helix connecting S4 and S5 in domain II (IIL45) and domains IIS5, IIS6, and IIIS6,

and the second binding site (PYR-2) is formed by the linker helix connecting S4 and S5 in domain I (IL45) and domains IS5, IS6, and IIS6 [29, 33, 97, 98].

In Mexico, pyrethroid-resistant *Ae. aegypti* populations carry at least two *vgsc* mutations, V1,016I which is linked to permethrin survival [74] and F1,534C. Interestingly, F1,534C reduces permethrin binding to *vgsc* channels whereas V1,016I has no effect in pyrethroid binding [29]. However, both replacements have co-evolved in Mexican populations; V1,016I and F1,534C allele frequencies have increased in the last 16 years [67] and F1,534C has reached fixation in several locations in Southern Mexico[99]. The co-occurrence of V1,016I and F1,534C has been reported in several countries in Latin America, including Brazil [100, 101], Venezuela [70], Colombia [102], Martinique Island [103], Puerto Rico [104], Grand Cayman Islands [68], Cuba [105], Haiti [106] and Jamaica [107].

Recently, a novel mutation V410L in domain IS6 was identified in a pyrethroid resistant laboratory strain of *Ae. aegypti* from Brazil [96]. Alone or in combination with F1,534C, V410L reduced the sensitivity of mosquito sodium channels expressed in *Xenopus* oocytes to both type I (eg. permethrin) and type II pyrethroids (eg. deltamethrin). Interestingly, the authors did not detect this mutation in field populations from Pernambuco, Brazil, concluding the novel mutation may not yet be widespread in nature. In contrast, we identified high frequencies of V410L alongside V1,016I and F1,534C in a genome-wide association study of *Ae. aegypti* from Mexico. To extend this observation, we screened the frequencies of V410L in different temporal and geographical *Ae. aegypti* collections made over the last 16 years in Mexico. We compared the evolution and linkage disequilibrium of V410L alongside the V1,016I and F1,534C replacements, which occur in different domains IS6, IIS6 and IIIS6, respectively (**Figure 3.1**). In addition, we

determined the phenotype/genotype association in a field population exposed to permethrin and deltamethrin.

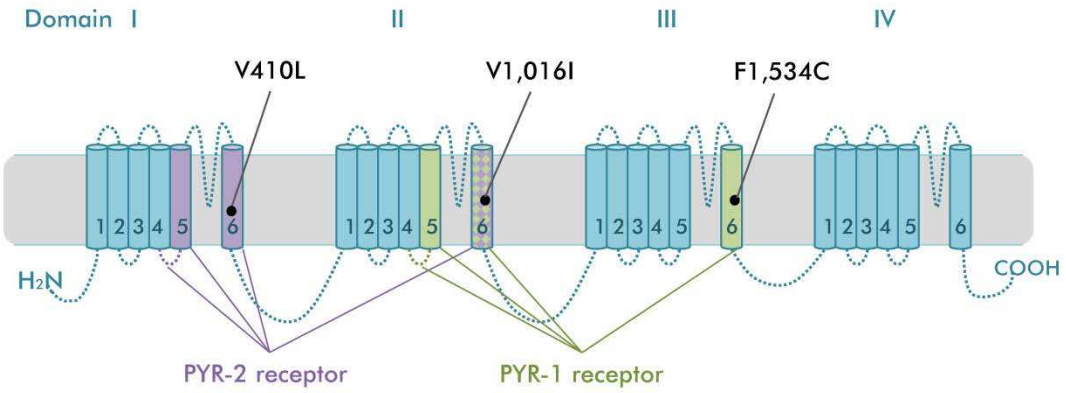


Figure 3. 1. Physical location of V410L, V1,016I and F1,534C replacements in the vgsc. The domain segments and inerlink helices forming the pyrethroid receptor sites 1 (green) and 2 (purple) are shown.

Methods

Detection of V410L and genotyping. Primer positions at exon 9 and 10 of *vgsc* in *Ae. aegypti* are shown in **Figure S3.1**. We used primers 410fw 5′-GATAATCCAAATTACGGGTATAC-3′ and 410rev 5′-TTCTTCCTCGGCGGCCTCTT-3′to amplify a ~500 bp region that flanked exon 9, intron 9–10 (~239 bp) and exon 10. PCR reactions were performed using 12.5 µl of GoTaq® Green (Promega, Madison WI), 11.65 µl H_20, 1.1 µl of each primer (at 50 pmol/µl) and 1 µl genomic DNA (~25 ng). PCR conditions were 3 min at 94 °C, followed by 35 cycles of 30 sec at 94 °C, 30 sec at 60 °C and 1 min at 72 °C and a final extension time of 5 min at 72 °C. Products were purified using MinElute® PCR purification columns (Qiagen, Hilden Germany) and sequenced using the primers 410_ex10fw 5′-TACGATCAGCTGGACCGTGG and 410rev targeting a fragment of 174 bp in exon 10. Sequences were analyzed using Geneious© software (Biomatters Inc, Newark NJ).

Following identification of the G (GTA=Val) and T (TTA=Leu) alleles at locus 410, we designed an allele-specific PCR system to detect individual genotypes by melting curve analysis [74]. Each reaction contained 50 µM of each forward primer V410fw (5′-GCGGGCAGGGCGGCGGGGGCGGGGCCATCTTCTTGGGTTCGTTCTACCGTG-3′), and L410fw (5′-GCGGGCATCTTCTTGGGTTCGTTCTACCATT-3′) and 100 µM of reverse primer 410rev (5′-TTCTTCCTCGGCGGCCTCTT-3′), 10 µl Sybr Green Master (BioRad, Hercules CA), 9.7 µl ddH_20 and 1 µl of genomic DNA (~25 ng). PCR and melting curve analysis was run in a CFX-96 (BioRad) following 3 min at 95 °C, 39 cycles of 10sec at 95 °C, 10sec at 60 °C, 30sec at 72 °C followed by a melting curve from 65 °C to 95 °C with increments of 0.2 °C during 10 sec. The products consisted of a single 133 bp amplicon for a VV_{410} homozygote, a single 113 bp amplicon for a LL_{410} homozygote (resistant) and the presence of both amplicons in heterozygote individuals (VL_{410}) (**Figure S3.2**).

Mosquito collections. We determined the V410L genotypes for 1,176 mosquitoes collected from six field locations in Mexico from 2000–2016 (**Figure 3.2** and **Table S3.1**). Four of these sites were in the State of Veracruz (Eastern Mexico). Tapachula is in the State of Chiapas in Southwestern Mexico and borders Guatemala, while Merida is in the Yucatan peninsula in Southeastern Mexico. The V410L database was completed with genotypes at loci 1,016 and 1,534 [99, 104].

Allele frequencies and linkage disequilibrium analysis. V410L allele frequencies were calculated from genotypic frequencies following Garcia *et al*.[67]. The 95% high density intervals (HDI 95%) around these frequencies were calculated in WINBugs©2.0 following 1,000,000 iterations. Departure from Hardy-Weinberg expectations were expressed as Wright's inbreeding

coefficient (F_{IS}) and a χ^2 test was used to test the hypothesis $F_{IS} = 0$ (d.f. = 1). Pairwise linkage disequilibrium between alleles at loci 410 and 1,016 or between loci 410 and 1,534 was calculated using LINKDIS following Vera-Maloof *et al*.[99]. Composite disequilibrium between resistant alleles was tested and a χ^2 test determined if significant disequilibrium existed among alleles at both loci.

Figure 3. 2. Locations of six *Aedes aegypti* collections in Mexico.

V410L association with pyrethroid resistance. To determine phenotype/genotype associations, we used the Viva Caucel (long −89.71827, lat 20.99827) collection from Yucatan, Mexico made in 2011. First, we calculated the permethrin and deltamethrin (Chem Service, West Chester PA)

lethal concentration that killed 50% of the mosquitoes (LC_{50}) in 3–4 days old adults of the F_3 generation. The insecticide treatment consisted of a 1 h exposure in an insecticide coated bottle, transfer of mosquitoes to recovery chambers and mortality scored at 24 h after treatment. We assessed the levels of permethrin and deltamethrin resistance by calculation resistance ratio (RR) in Viva Caucel relative to the New Orleans (NO) reference strain. The permethrin LC_{50} was 47.9-fold higher than NO (26.5 µg vs 0.55 µg, respectively) and the deltamethrin LC_{50} was 47.6-fold higher than NO (10.49 µg vs 0.22 µg).

Once the LC_{50} was calculated, six to 10 groups of 50 mosquitoes at a time were exposed to 25 µg of permethrin or 3 µg of deltamethrin coated in 250 mL Wheaton bottles during 1 h. Immediately following exposure, active and inactive mosquitoes were transferred to separate containers. To ensure correct categorization, we phenotyped mosquitoes 4 h after treatment. We observed activity and if the mosquitoes were capable of flight, they were scored as 'alive group'. In the inactive group we separated the newly recovered mosquitoes from the inactive mosquitoes and scored them as 'recovered' and 'dead', respectively. **Table S3.2** shows the total number of mosquitoes exposed to permethrin and deltamethrin and the distribution between the three phenotypic categories.

A subsample of mosquitoes from each group was individually frozen; DNA was isolated by the salt extraction method [72] and resuspended in 150 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0). For the Viva Caucel mosquitoes exposed to permethrin we randomly selected 95 knockdown-resistant, 95 recovered and 95 dead mosquitoes. For deltamethrin we randomly selected 111 knockdown-resistant, 67 recovered and 92 dead mosquitoes. We conducted genotyping at locus 410 using the V410L melting curve system described above. For V1,016I and F1,534C genotypes, we used previously described methodologies [69, 74]. A

contingency table was used to test for association between the phenotypes (alive, recovered and dead) and genotypes (mutant homozygote, wild type homozygote, and heterozygote) at each locus separately (410, 1,016 and 1,534) and for the 27 (3 ×3 ×3) tri-locus genotype combinations.

Results

V410L in *Ae. aegypti* collections from Mexico. We determined the V410L genotypes for 1,176 mosquitoes collected from six field locations in Mexico from 2000–2016. The V410L genotype counts and calculated allele frequencies are shown in **Table 3.1**. V410L first appeared in a heterozygous individual mosquito in 2002 in Coatzacoalcos. By 2008, L410 allele frequencies ranged from 0.27–0.65 across collections. By 2012, L410 frequencies ranged from 0.56 to 0.84 in collections from the State of Veracruz. By 2014, Tapachula and Merida had allele frequencies of 0.57 and 0.9, respectively. V410L genotype frequencies were in Hardy-Weinberg equilibrium in 13 out of 14 collections where V410L was segregating. The exception was in Coatzacoalcos 2008 that had a significant deficiency of LL_{410} homozygotes (F_{IS}=−0.382). **Figure 3.3** shows the allele frequencies of L410, I1,016 and C1,534 at four-time points: 2000 (n=233), 2002–2005 (n= 346), 2006–2008 (n= 223) and 2012–2016 (n= 374) for all locations. As previously observed for I1,016 and C1,534 [99], L410 also increased in frequency from 2000 to 2016, noting that at each of these time points L410 and I1,016 alleles changed frequencies in parallel from 0.00 to 0.71 (**Figure 3.3**).

Table 3. 1. V410L genotypes and allele frequencies in 25 collections form Mexico. The site, year, sample size, genotype frequency, L410 allele frequency, 95% high density intervals (HDI) and inbreeding coefficients (FIs). L=resistant allele, V= susceptible allele.

			V410L genotype			L410 allele frequency and 95% HDI			
Site	**Year**	**n**	**VV**	**VL**	**LL**	**Freq.**	**lower**	**upper**	**F_{IS}**
Poza Rica									
	2000	46	46	0	0	0.00	0.0003	0.04	
	2003	47	47	0	0	0.00	0.0003	0.04	
	2008	39	5	17	17	0.65	0.5400	0.75	0.04
	2012	37	3	12	22	0.76	0.6476	0.84	0.12
Martinez de la Torre									
	2000	46	46	0	0	0.00	0.0003	0.04	
	2002	42	42	0	0	0.00	0.0003	0.04	
	2003	30	24	5	1	0.12	0.0586	0.22	0.19
	2008	48	9	25	14	0.55	0.4521	0.65	−0.05
	2012	44	0	14	30	0.84	0.7555	0.9	−0.18
Zempoala									
	2000	47	47	0	0	0.00	0.0003	0.04	
	2002	47	47	0	0	0.00	0.0003	0.04	
	2003	30	30	0	0	0.00	0.0004	0.06	
	2012	52	5	18	29	0.73	0.6246	0.79	0.12
Coatzacoalcos									
	2002	50	48	2	0	0.02	0.0062	0.07	−0.02
	2003	48	48	0	0	0.00	0.0003	0.04	
	2008	48	21	26	0	0.27	0.1964	0.37	−0.38
	2012	45	9	22	14	0.56	0.4626	0.66	0.01
Tapachula									
	2000	48	48	0	0	0.00	0.0003	0.04	
	2006	42	28	11	3	0.20	0.1307	0.3	0.19
	2014	47	7	26	14	0.57	0.4732	0.67	−0.13
	2016	96	15	40	41	0.64	0.5653	0.7	0.1
Merida									
	2000	47	47	0	0	0.00	0.0003	0.04	
	2005	48	48	0	0	0.00	0.0003	0.04	
	2007	47	7	30	10	0.53	0.4315	0.63	−0.28
	2013	50	0	10	40	0.90	0.8256	0.94	−0.11
New Orleans									
		48	48	0	0	0.00			

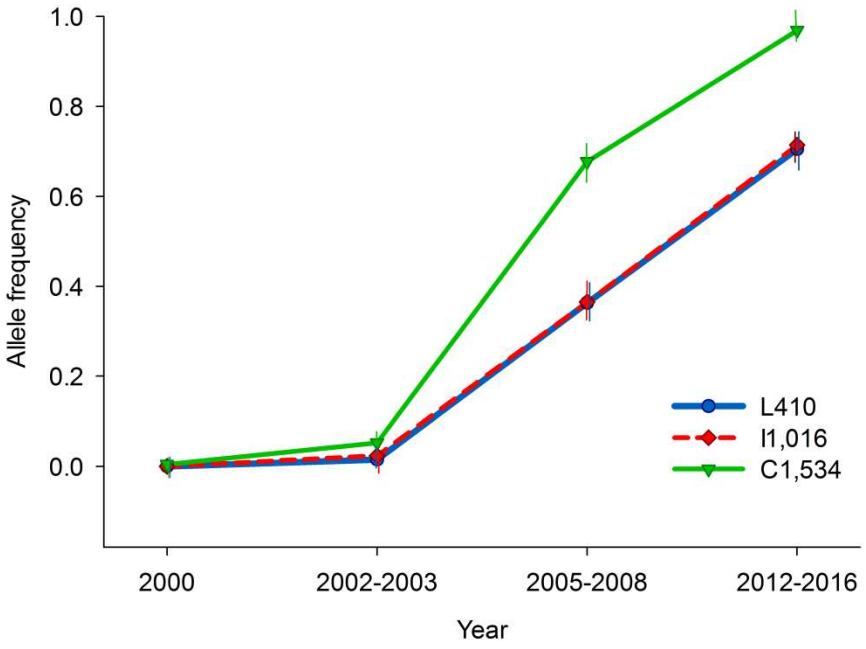


Figure 3. 3. L410, I1,016 and C1,534 allele frequencies in 25 *Ae. aegypti* collections form Southern Mexico. Allele frequencies are plotted in four periods of time: 2000 (n=233), 2002-2005 (n=346), 2006-2008 (n=223) and 2012-2016 (374).

Pairwise linkage disequilibrium analyses were performed between locus 410-1,016, 410–1,534 and 1,016– 1,534. **Table 3.2** shows the linkage disequilibrium coefficients (R_{ij}), χ^2 and associated probabilities obtained between pairwise loci. Fourteen out of 25 collections had alleles segregating at loci 410–1,016 with R_{ij} values ranging between 0.53–0.99 among collections; overall R_{ij} was 0.96 (p =0.0001). For loci 410–1,534, only five collections had mutant alleles segregating, and four were in significant linkage disequilibrium with R_{ij} ranging from 0.33 to 0.99; the overall R_{ij} coefficient was 0.76 (p =0.0001). At loci 1,016–1,534, segregating alleles from six collections were in significant linkage disequilibrium, with R_{ij} coefficients ranging from 0.31 to 0.99; overall R_{ij} coefficient was 0.76 (p=0.0001).

Table 3. 2. Linkage disequilibrium coefficients (R_{ij}), χ^2 and associated probabilities between loci 410-1,016, 410-1,534 and 1,016-1,534.

		410-1,016			410-1,534			1,016-1,534		
Site	**Year**	R_{ij}	χ^2	***P***	R_{ij}	χ^2	***P***	R_{ij}	χ^2	***P***
Poza Rica										
	2008	0.67	17.3	0.0001	0.41	6.5	0.0106	0.72	20	0.0001
	2012	0.61	13.9	0.0002	0.31	3.5	0.0622	—	—	—
Martinez de la Torre										
	2003	0.53	8.5	0.0035	—	—	—	0.69	14.3	0.0002
	2008	0.99	48	0.0001	—	—	—	—	—	—
	2012	0.99	45	0.0001	—	—	—	—	—	—
Zempoala										
	2012	0.81	34.3	0.0001	—	—	—	—	—	—
Coatzacoalcos										
	2002	0.99	50	0.0001	0.99	50	0.0001	0.99	50	0.0001
	2008	0.99	47	0.0001	—	—	—	—	—	—
	2012	0.99	47	0.0001	—	—	—	—	—	—
Tapachula										
	2006	0.99	42	0.0001	0.33	4.7	0.0303	0.33	4.7	0.0303
	2014	0.89	37.3	0.0001	—	—	—	—	—	—
	2016	0.99	96	0.0001	—	—	—	—	—	—
Merida										
	2007	0.88	36	0.0001	0.96	43.4	0.0001	0.82	31.4	0.0001
	2013	0.82	33.9	0.0001	—	—	—	0.31	4.8	0.0278
Overall population		0.96	1081.7	0.0001	0.76	675.9	0.0001	0.76	676.9	0.0001

Temporal analysis of tri-locus genotypes. Out of 27 genotype combinations (3 genotypes at 3 loci), we found 20 tri-locus genotype combinations in 1,176 individual mosquitoes collected from 2000 to 2016. **Figure 3.4** shows the frequency of each of the 20 tri-locus genotype combinations at four-time points: 2000, 2002–2005, 2006–2008 and 2012–2016. In 2000, the triple homozygote susceptible genotype ($VV_{410}/VV_{1,016}/FF_{1,534}$) occurred at a high frequency (0.99) whereas a genotype including a heterozygote at loci 1,534 ($VV_{410}/VV_{1,016}/FC_{1,534}$) had a frequency lower than 0.01 (**Figure 3.4A**). By 2002–2005, these genotypes were still the most common (frequencies of 0.86 and 0.08, respectively); however, six additional genotypes including homozygotes at locus 1,534 and heterozygotes at loci 1,016 and 410 occurred at very low

frequencies (<0.02) (**Figure 3.4B**). By 2006–2008, the triple homozygote susceptible genotype ($VV_{410}/VV_{1,016}/FF_{1,534}$) and the 1,534 heterozygotes decreased to frequencies lower than 0.06. Twelve additional combinations occurred, the four most frequent genotypes were $VV_{410}/VV_{1,016}/CC_{1,534}$ (frequency=0.2), $VL_{410}/VI_{1,016}/FC_{1,534}$ (0.18), $VL_{410}/VI_{1,016}/CC_{1,534}$ (frequency=0.26) and the triple resistant homozygote $LL_{410}/II_{1,016}/CC_{1,534}$ (frequency=0.18) (**Figure 3.4C**). Observed frequencies of these genotypes were significantly higher than expected (**Table S3.2**). By 2012–2016, the triple homozygote susceptible genotype was no longer detected, and the most common genotype combinations were the triple resistant homozygote (frequency=0.47) and $VL_{410}/VI_{1,016}/CC_{1,534}$ (frequency=0.34) (**Figure 3.4D**). Observed frequencies of these genotypes were in significant excess. In the same period, very low frequencies of resistant homozygotes at locus 410 occurred independently of 1,016 ($LL_{410}/VV_{1,016}/CC_{1,534}$ or $VV_{410}/II_{1,016}/CC_{1,534}$ and $LL_{410}/VV_{1,016}/FC_{1,534}$ or $VV_{410}/II_{1,016}/FC_{1,534}$). Also, genotypes including heterozygotes at locus 410 and homozygotes at locus1,016 and vice versa ($VL_{410}/II_{1,016}/CC_{1,534}$ or $VL_{410}/II_{1,016}/FC_{1,534}$ and $LL_{410}/VI1_{,016}/CC_{1,534}$ or $LL_{410}/VI_{1,016}/FC_{1,534}$) were observed at 10-fold lower frequencies (~7 individuals) than expected (**Table S3.3**).

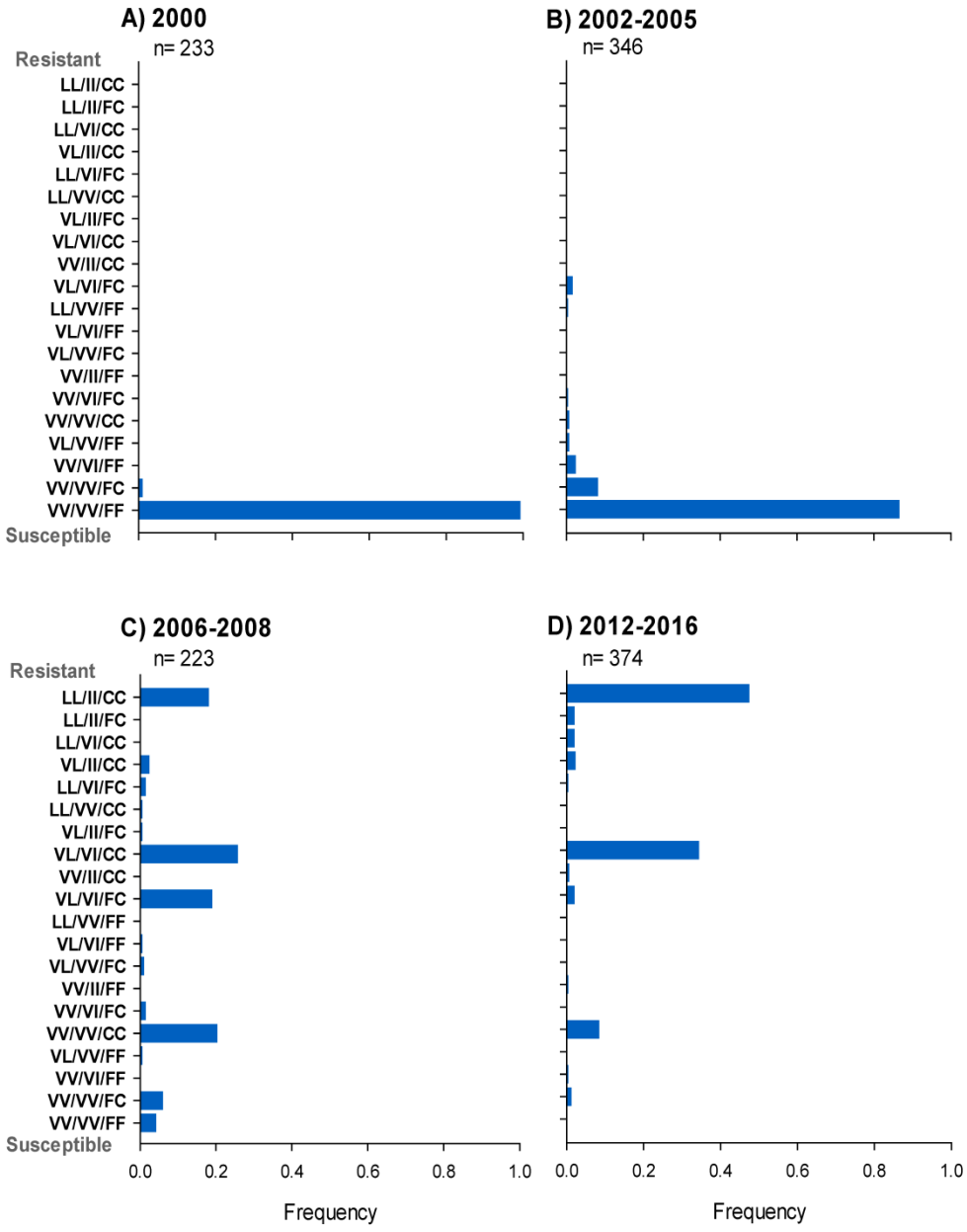


Figure 3. 4. Frequencies of the 20 tri-locus genotypes plotted by periods of time. (A) Frequencies in 2000, (B) Frequencies in 2002-2005, (C) Frequencies in 2006-2008 and (D) Frequencies in 2012-2016. The order of the genotypes is 410/1,016/1,534. Resistant allele at 410=L, 1,016=I and 1,534=C. The triple susceptible genotype is at the bottom of each graph whereas the triple resistant genotype is at the top of each yaxis.

Association of V410L with pyrethroid resistance. L410 was present at a frequency of 0.69 in Viva Caucel mosquitoes used for our phenotype/genotype association study. We used a dose of 25 µg/bottle permethrin or 3µg/bottle deltamethrin to discriminate three phenotypes: alive, recovered and dead. The susceptible genotype at locus 410 was VV_{410}, heterozygote was VL_{410} and resistant was LL_{410}. **Table 3.3** shows the outcomes of mosquitoes carrying a specific genotype in terms of response to pyrethroid treatment. For permethrin, 53% of the resistant homozygotes (LL_{410}) were alive, 40% recovered and only 7% died. In the heterozygote group (VL_{410}) 4% was alive, 28% recovered and 64% died following permethrin exposure. For deltamethrin, 72% of the resistant homozygotes (LL_{410}) were alive whereas 23% recovered and only 5% died. Note that the phenotype outcome is very similar between genotypes at loci 410 and 1,016. The most striking difference at locus 1,534, is that more than 92% of the heterozygotes died after exposure to permethrin or deltamethrin. Across all analyses, strong correlations were detected between phenotype and genotype.

Table 3. 3. Phenotype and genotype at loci 410, 1,016 and 1,534 analyzed separately in mosquitoes from Viva Caucel treated with permethrin or deltamethrin. The percentage of knockdown resistant, recovered and dead mosquitoes within the genotype group is shown in parenthesis. The *p* value corresponds to a 3 x 3 table contingency analysis performed for each locus.

Loci	Genotype	Permethrin			Deltamethrin		
		Knockdown resistant	Recovered	Dead	Knockdown resistant	Recovered	Dead
		n= 94	n= 95	n= 95	n= 111	n= 67	n= 92
V410L							
	LL	87 (53%)	66 (40%)	12 (7%)	86 (72%)	28 (23%)	6 (5%)
	VL	4 (5%)	28 (32%)	56 (64%)	24 (20%)	35 (29%)	52 (47%)
	VV	2 (7%)	1 (3%)	27 (90%)	1 (1%)	4 (3%)	34 (87%)
	p	4.0 × 10−30			6.1 × 10−26		
V1,016I							
	II	90 (54%)	66 (40%)	11 (7%)	86 (70%)	27 (22%)	9 (7%)
	VI	3(3%)	28 (32%)	57 (65%)	24 (20%)	36 (30%)	52 (46%)
	VV	1 (3%)	1 (3%)	27 (93%)	1 (1%)	4 (3%)	31 (86%)
	p	1.4 × 10−32			1.2 × 10−23		
F1,534C							
	CC	90 (37%)	95 (39%)	58 (24%)	111 (56%)	65 (33%)	24 (12%)
	FC	3 (8%)	0 (0%)	36 (92%)	0 (0%)	2 (1%)	61 (97%)
	FF	1 (50%)	0 (0%)	1 (50%)	0 (0%)	0 (0%)	6 (100%)
	p	7.9 × 10−15			6.8 × 10−35		

Association of tri-locus genotypes with pyrethroid resistance. Because our results indicated that L410, I1,016 and C1,534 do not occur independently, we analyzed the phenotype outcome by tri-locus genotype combinations. In the Viva Caucel population, 13 tri-locus genotype combinations were identified. **Figure 3.5** shows the distribution of knockdown resistant, recovered and dead mosquitoes for the eight most common tri-locus genotype combinations. The presence of resistant alleles in the tri-locus homozygote genotype is strongly associated with alive and recovery phenotype for both permethrin and deltamethrin (**Figure 3.5A, B**). Wild type homozygotes at locus 410 and 1,016 in presence of $FC_{1,534}$ or $CC_{1,534}$ were associated with the dead phenotype. The double heterozygotes at locus 410 and 1,016 in presence of $FF1_{,534}$ or

$FC_{1,534}$ were also associated with the dead phenotype for both pyrethroids. The double heterozygote at locus 410 and 1,016 with $CC_{1,534}$ (VL_{410}/$VI_{1,016}$/ $CC_{1,534}$) was associated with the dead phenotype for permethrin exposure but was associated with alive phenotype and recovery phenotype in the deltamethrin exposure group.

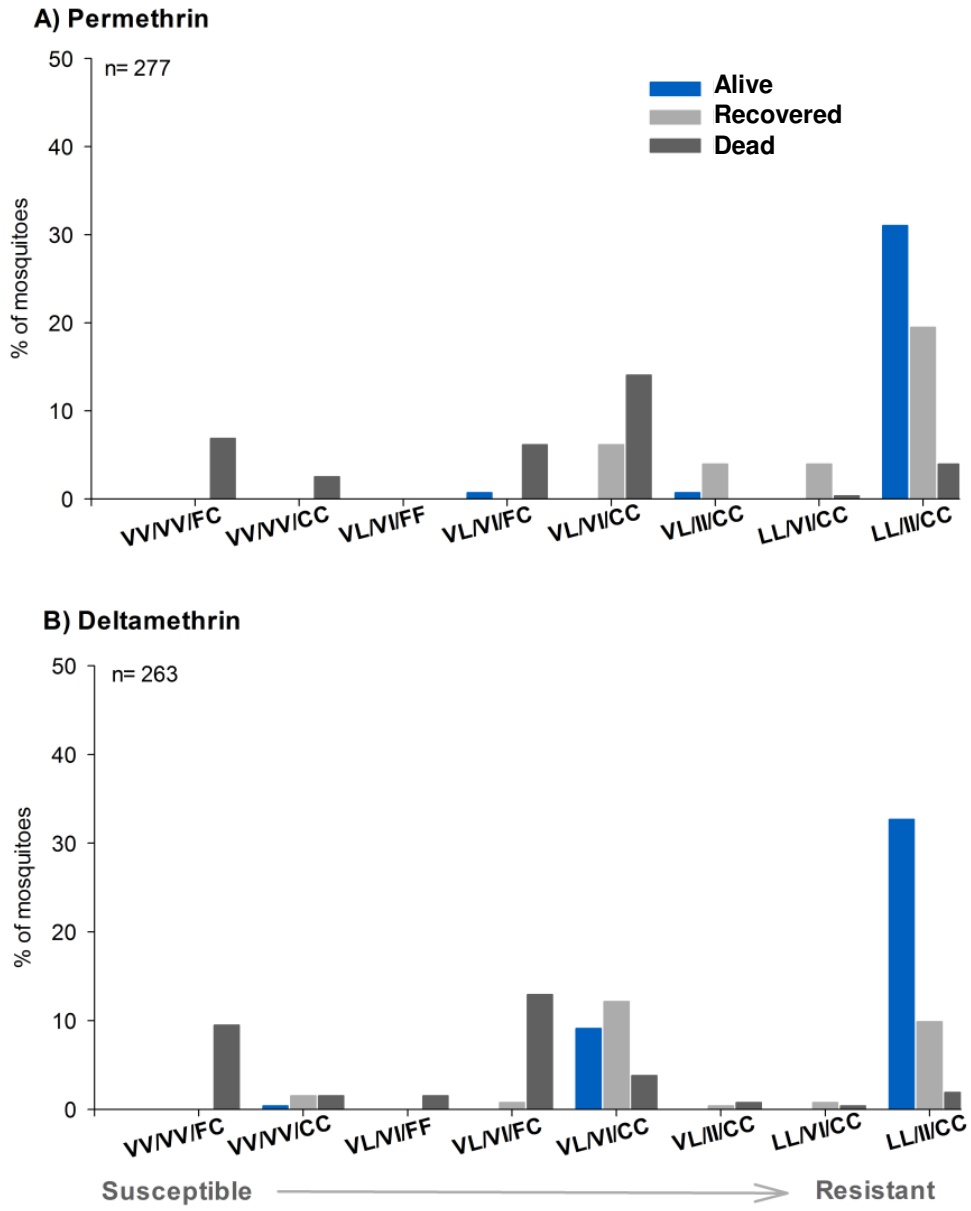


Figure 3. 5. Frequencies of tri-locus genotypes in alive, recovered and dead mosquitoes following (A) permethrin or (B) deltamethrin exposure. The order of the genotypes is shown for locus 410/1,016/1,534 respectively. Resistant allele at loci 410=L, 1,016=I and 1,534=C. The triple susceptible genotype is closer to the y axis whereas the triple resistant genotype is shown on the far right side of the x axis.

Discussion

Different replacements at residue V410 have been reported in the *vgsc* of several pyrethroid resistant insect species. Specifically, V410L was associated with deltamethrin resistance in the common bed bug *Cimex lectularis* [108]. However, replacements V410M in the tobacco budworm *Heliothis virescens* [109] and V410A, V410G and V410M in the earworm *Helicoverpa zea* [110] have also been reported. In *Ae. aegypti*, V410L was recently detected in a pyrethroid resistant insectary strain from Brazil [96], which demonstrated that V410L alone or in combination with the F1,534C reduced the sensitivity of mosquito sodium channels expressed in *Xenopus* oocytes to both type I and II pyrethroids. In the same study, V410L was not detected in a small field survey in the State of Pernambuco, Brazil, and authors suggested that V410L was not yet widespread in the field. Importantly, our results show that V410L has existed for at least 16 years in Mexico, the first heterozygote was detected in 2002 in Coatzacoalcos, Veracruz, and has increased gradually to high frequencies in 2016 (up to 0.92). Interestingly, we found L410 to be in greater linkage disequilibrium with I1,016 than with C1,534. Our previous study measured linkage disequilibrium between I1,016 and C1,534 and we proposed a sequential model, wherein C1,534 first occurred (providing low levels of resistance) and then the replacement I1,016 occurred with this haplotype, providing even higher levels of resistance. V410L challenges this sequential model, in which both V410L and V1,016I might have occurred independently on a C1,534 haplotype and then became *cis* to C1,534 by recombination. An alternative model assumes the three mutations arose independently at very low frequencies; and then, by two recombination events, came to occur in a *cis* arrangement. Our results indicate that C1,534 was at a frequency of 0.004 in collections from 2000 whereas L410 and I1,016 were below limits of detection. By 2002–2005, C1,534 was at higher frequency (0.055), while L410 and I1,016

appeared at lower frequencies (0.013 and 0.02, respectively). During this period, we identified heterozygotes at 410 and 1,016 occurring independently in a $FF_{1,534}$ background. Also, the triple heterozygote was identified in low frequencies (5/346). By 2006–2008 heterozygotes VL_{410} and $VI_{1,016}$ on a $FC_{1,534}$ heterozygote or $CC_{1,534}$ homozygote were favored by selection, whilst mutant genotypes at 410 and 1,016 never occurred independently or were at very low frequencies.

A recent study found that V410L and F1,534C occurred without V1,016I in a deltamethrin resistant laboratory strain originated from Rio de Janeiro [96]. In contrast, we show large linkage disequilibrium between L410 and I1,016, except among very few individuals collected early in 2002–2005. This genotype combination was no longer detected in following years. Whether L410 remains independent of I1,016 in Brazil will provide evidence of the mutations arising independently at a local level in the sequential model. However, I1,016 and C1,534 are already widespread in several regions of Brazil, and due to high migration rates among *Ae. aegypti* populations, we would expect I1,016 and C1,534 to recombine with L410-C1,534 in future years. An alternative scenario is that, as in Mexico, L410 is already present at high frequencies in Brazilian collections previously genotyped with I1,016-C1,534 but simply has not yet been detected.

The selection of the triple homozygote resistant genotypes detected in our data suggests higher fitness of this genotype in the presence of pyrethroids. The role of L410 and C1,534 in conferring pyrethroid resistance was determined in Haddi *et al*. 2017 [96]. L410 alone or in combination with C1,534 confers high levels of resistance, however, it remains to be seen if it is fit in the field. We found 4 out of 1,176 individuals with L410 and C1,534 occurring independently (in the absence of I1,016), and this genotype became extinct in Mexican populations. In our phenotype and genotype studies, the triple homozygote resistant individuals

had better survival (alive and recovery) following either permethrin or deltamethrin exposure. One particular genotype, $VL_{410}/VI_{1,016}/CC_{1,534}$ had different outcome depending of the specific pyrethroid, with this genotype associated with dead in mosquitoes following permethrin exposure. In contrast, this genotype was mostly associated with survival (knockdown and recovery) in mosquitoes exposed to deltamethrin. Apparently, the presence of heterozygotes at loci 410 and 1,016 was sufficient for deltamethrin survival.

F1,534C is located in the PYR-1 receptor site and is responsible for reducing vgsc sensitivity to permethrin. Although residue 1,016 is located in the PYR-1 receptor site, only the V1,016G replacement occurring in *Ae. aegypti* from Asia reduces vgsc whilst the V1,016I replacement found in the Americas does not [98]. In contrast, V410L is located in DIS6 but does not form part of the PYR-2 receptor site. It has been suggested that the reduction of sodium channel sensitivity to permethrin and deltamethrin by V410L might result from changes in the gating properties of vgsc without inhibiting molecule docking [96]. Because pyrethroids prefer to bind to sodium channels in the open state, kdr mutations that deter the open state would counteract the pyrethroid effects [96]. In recent structural models, pyrethroids make multiple contacts with helices IIL45, IIS5, IIS6, and IIIS6, as well as IL45, IS5, IS6, and IIS6 that would maintain vgsc in an open state [30, 33]. Simultaneous binding of pyrethroids to both PYR-1 and PYR-2 is thought to effectively prolong the opening of vgsc [29]. It is possible that co-occurrence of V410L and V1,016I, although in different receptor sites, provide fitness advantages in the presence of pyrethroids, thus favoring co-selection. The interaction of both mutations in electrophysiology experiments will address if the co-occurrence of these mutations is compensatory or synergistic in the presence of pyrethroids.

Chapter 4: Loss of pyrethroid resistance in newly established laboratory colonies of *Aedes aegypti*.

Introduction

After almost two decades of frequent pyrethroid use for *Aedes aegypti* (L.) control there is now widespread pyrethroid resistance in Mexico [111]. A key component of resistance management assumes that there will be a negative fitness associated with resistance alleles so that when insecticides are removed, resistance alleles will decline in frequency. Laboratory strains of *Aedes aegypti* have shown a decrease of insecticide resistance once the insecticide is removed suggesting a fitness cost associated with resistance [1, 2, 112-114]. To date, three studies have evaluated the loss of pyrethroid resistance in *Ae. aegypti*. In Taiwan, a permethrin-resistant laboratory strain was maintained for 47 generations under permethrin pressure. Following 15 generations without exposure there was a significant decrease in the permethrin resistance ratio (RR) and the resistant alleles (G1,023 and Y1,794) [114]. In Brazil, after 15 generations, the frequency of I1,016 decreased from 0.75 to 0.20 [1]. A study in Mexico, showed that despite no evident change in the frequency of resistant alleles (I1,016 and C1,534) after 10 generations following removal of pyrethroids, there was a significant increase in the proportion of knocked-down mosquitoes [113].

The present study aims to evaluate the loss of pyrethroid resistance from eight field populations of *Ae. aegypti*, (six field collections from or near the city of Merida and two collections from Tapachula and Acapulco from southern Mexico) to assess variation in the rate of loss of pyrethroid resistance. These collections were maintained for up to eight generations after pyrethroids were discontinued. We recorded changes in the frequencies of two *kdr* mutations I1,016 and C1,534, and the analysis of resistance ratios with permethrin (pyrethroid

type 1) and deltamethrin (pyrethroid type 2) RR. In generations F_3, F_6, and F_8 we also evaluated fecundity to test for parallel changes in a fitness trait during the eight generations. This was also analyzed because a negative correlation between resistance and fecundity has previously been described in two studies [1, 2].

Methods

***Aedes aegypti* field populations.** In 2014 F_0 mosquito eggs were collected from eight sites in Mexico. The F_0 Eggs were hatched in the laboratory and were reared to adulthood to be identified and separated by species. These adult *Ae. aegypti* were maintained for each separate colony in the lab and produced F_1 eggs. The location of the collection sites appear in **Figure 4.1** and GPS coordinates and name abbreviations appear in **Table 4.1**. Eggs from Yucatan were collected from three sites in urban areas of Merida and three collections from villages near Merida. Mosquito eggs from Chiapas and Guerrero were located in Tapachula and in Acapulco, respectively.

Establishment and maintenance of field populations. F_1 eggs were sent to Colorado State University. Eggs papers were placed into water containers with 2 L of tap water to promote development and hatching. Larvae were fed with 2 mL of 10% liver powder (10 gm of liver powder suspended in 100 mL of water) every other day. Each collection was split at the larval stage into three subgroups to generate three biological replicates. We transferred the pupae to plastic cages for mosquito emergence. Larval and adult mosquitoes were maintained in an incubator at 27-28^{o} C, 70-80% humidity, and a photoperiod of 12h light: 12h dark. Adults were fed with raisins and allowed access to tap water. Females were offered citrated sheep blood

(Colorado Serum Company, Denver CO) on artificial feeders, every four days, to obtain eggs. Females laid their eggs on moistened filter paper. We let the eggs develop for 48 hrs before they were partially dried at room temperature and stored in sealed plastic bags. At least 1,000 adults were used to continue the population from one generation to the next.

Figure 4.1. Geographic locations of *Aedes aegypti's* collection sites from Southern Mexico.

Table 4.1. States and cities of collection sites, geographical coordinates and site's abbreviations used in this study.

State	City	Population size	Site	Latitude	Longitude	Abbreviation
Guerrero						
	Acapulco	810,669	Zapata	16.9049222	-99.8410944	**Acp**
Chiapas						
	Tapachula	348,156	Col.5 de Febrero	14.9204944	-92.2593472	**Tap**
Yucatan						
	Merida	892,363	Fco. Montejo	21.0307194	-89.6463639	**Mer1**
			Plan Ayala	21.0135833	-89.6222222	**Mer2**
			U.H. Morelos	20.9420139	-89.5981556	**Mer3**
	Conkal	11,141	Center	21.0747917	-89.5199056	**Co**
	Dzitya	2,000	Center	21.0623278	-89.6746694	**Dz**
	Acanceh	16,127	Center	20.8126083	-89.4505611	**Ac**

Genotyping V1,016I and F1,534C. DNA was extracted, at each generation (F_1-F_8), from individual mosquitoes by the salt extraction method [72] and suspended in 180 µL of TE buffer (10 ml of 1 M Tris-HCI pH 8.0, 2 ml of 0.5 M EDTA pH 8.0 brought to 500 mL with distilled water). To identify allelic variation we used allele-specific polymerase chain reaction (as PCR), followed by generation of a melting curve approach (CFX-96 BioRad), to identify genotypes [69, 73, 74]. In each of the eight generations, we analyzed three replicates of 50 adult mosquitoes (~25♀ and 25♂) for each of the eight collection sites. Collection sizes were intentionally large to minimize founder's effect and genetic drift.

Allele frequencies and linkage disequilibrium analysis. We estimated the allele frequencies of each of the eight generations from the genotypic frequencies (resistant allele = ((2* resistant homozygote) + heterozygote) / 2N). We used WINBugs©2.0 [79] with 1,000,000 iterations to calculate the 95% high-density intervals (HDI 95%) around the allele frequencies. Wright's inbreeding coefficient (F_{IS}) and a $\chi 2$ test were used to test the hypothesis $F_{IS} = 0$ (genotypes in

Hardy-Weinberg Equilibrium (HWE)) (d.f.=1). We used R©-3.5.1 to graph the data. In addition, we used LINKDIS [76] and χ^2 test to calculate the pairwise linkage disequilibrium between alleles at loci 1,016 and 1,534 [99].

Quantification of pyrethroid resistance ratio. To determine the level of resistance to permethrin (type 1 pyrethroids) and deltamethrin (type 2) following the removal of pyrethroids, we evaluated generations F_3, F_6, and F_8. The resistance ratio (RR) was determined from the concentration of an insecticide that killed 50% of mosquitoes in a bottle, relative to our susceptible New Orleans (NO) strain. Therefore, first, we calculated the permethrin and deltamethrin (Sigma-Aldrich, Cat # 45614 and 45423, respectively) LC_{50} of each population . Approximately, 15-25 female mosquitoes (3-5 day old) were exposed to a pyrethroid for one hour. Bottles were coated internally with at least five different concentrations of insecticide. Bottles coated with acetone were used as technical controls. Exposed mosquitoes were transferred to recovery cups and placed in an incubator at 27-28^o C and 70-80% of humidity. Mortality was scored at 24 h after treatment. We used QCal© to obtain the LC_{50} and the confidence intervals [115].

Evaluation of fecundity (eggs/female). We calculated the number of eggs laid by females in generations F_3, F_6 and F_8 to evaluate changes in fecundity in the eight collections after removal of pyrethroids. New Orleans was used as a reference strain. Six groups of five blood-fed and engorged females of 5-7 days old were placed into paper cups that contained a damp filter paper that acted as a substrate for oviposition. Mosquitoes were maintained at 27-28^o C and 70-80% humidity. Females that died during the experiment, before oviposition, were excluded from the analysis. Females were removed after 72 hrs of being blood fed. The numbers of eggs were

recorded 48 hrs after the eggs had melanized in humid conditions. Fecundity was the number of eggs placed in each cup divided by the number of females that laid eggs. This was the number of females alive when eggs were first noticed. Three replicates were performed, therefore, a total of 90 females were tested per collection site. In each generation we used Dunn's Multiple Comparison Test to evaluate differences in the average numbers of oviposited eggs among the eight field sites relative to NO susceptible. We used PROC CORR in SAS® 9.4 (SAS Institute, Cary NC) to calculate Pearson's correlation coefficient to examine the relationship between averaged numbers of eggs and frequencies of the resistant alleles (I1,016 and C1,534), and between eggs and the RR of permethrin and deltamethrin relative to NO.

Results

V1,016I in *Ae. aegypti* collections after removal of pyrethroid selection. We determined the V1,016I genotype of 9,563 mosquitoes from southern Mexico over eight generations (F_1-F_8) following removal from pyrethroid exposure. The V1,016I genotype counts and allele frequencies appear in **Table 4.1S and 4.2S**, respectively. **Figure 4.2A** plots I1,016 allele frequencies in all three replicates in the different collections over eight generations. In general, I1,016 allele frequencies were statistically uniform among replicates in the collections with five exceptions ($Mer3F_7$, DzF_2,CoF_2, CoF_8, AcF_2 - **Table 4.2S**). Genotypes at locus 1,016 were segregating in all 192 analyses and 24 of those were not in HWE (**Table 4.2S**). Sixteen of the 24 had an excess of heterozygotes ($VI_{1,016}$) and 8 had a deficiency of heterozygotes.

Figure 4.2B plots the average of I1,016 frequencies among all the three replicates and the correlation between generation number. Allele frequencies in F_1 ranged from 0.35-0.83 across collections. By F_8, I1,016 frequencies ranged from 0.31-0.63. The average across

populations of I1,016 allele frequency declined from 0.63 in generation F_1 to 0.42 in generation F_8.

In all collections I1,016 declined in frequency over eight generations. However, the rate and pattern of decline depended greatly upon the collection. Four out of the eight collection sites had a significant drop in I1,016 frequency from generation F_1 to F_8 (Ac, Acp, Co, and Mer2) (**Figure 4.2B**). Sites Dz, Mer1 and Tap only changed slightly over 8 generations. Collections Co, Mer3 and Tap declined initially and then, surprisingly, increased in the last 3-4 generations.

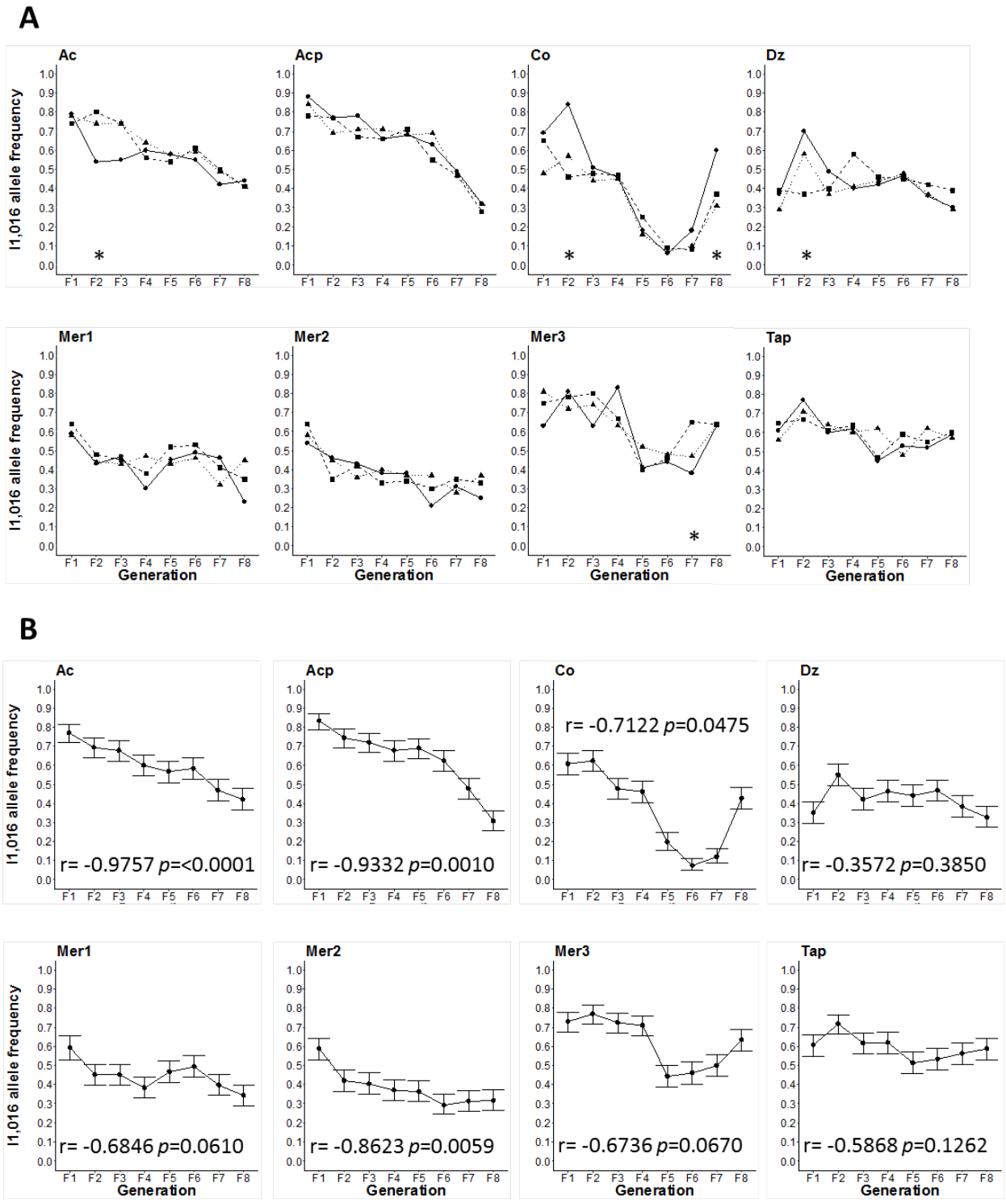


Figure 4.2. I1,016 allele frequencies across eight generation of pyrethroid removal in eight *Ae. aegypti* collection samples from Southern Mexico and Pearson´s correlation coefficient and probability. A) All replicates shown, * indicates significant differences between replicates and B) Average allelic frequencies, error bars represent 95% HDI.

Figure 4.3 shows the V1,016I genotype frequencies ($VV_{1,016}$, $VI_{1,016}$, $II_{1,016}$) over eight generations in each of the 8 collections. **Table 4.2** lists the Pearson correlations coefficients between genotype frequencies and generation number. The correlation coefficients for $VV_{1,016}$ were all positive and four were significant (Ac, Acp, Mer2, & Tap). Six of the correlation coefficients for $VI_{1,016}$ were positive but none were significant and all of the $II_{1,016}$ were negative and six were significant (Ac, Acp, Co, Mer1, Mer2, Mer3). Across all collection sites, there was a positive correlation (r = 0.4497, p=0.0002) between $VV_{1,016}$ frequencies and generation number. The frequencies of $VI_{1,016}$ heterozygotes did not change over the eight generations (r = 0.1462, P=0.2489) and were only significantly positive in Acp. As expected $II_{1,016}$ genotypic frequencies decreased significantly over generations (r = -0.5452, P<0.0001). There was a negative correlation between $II_{1,016}$ genotypic frequencies and generation in all collections and 6 of these were significant (**Table 4.2**).

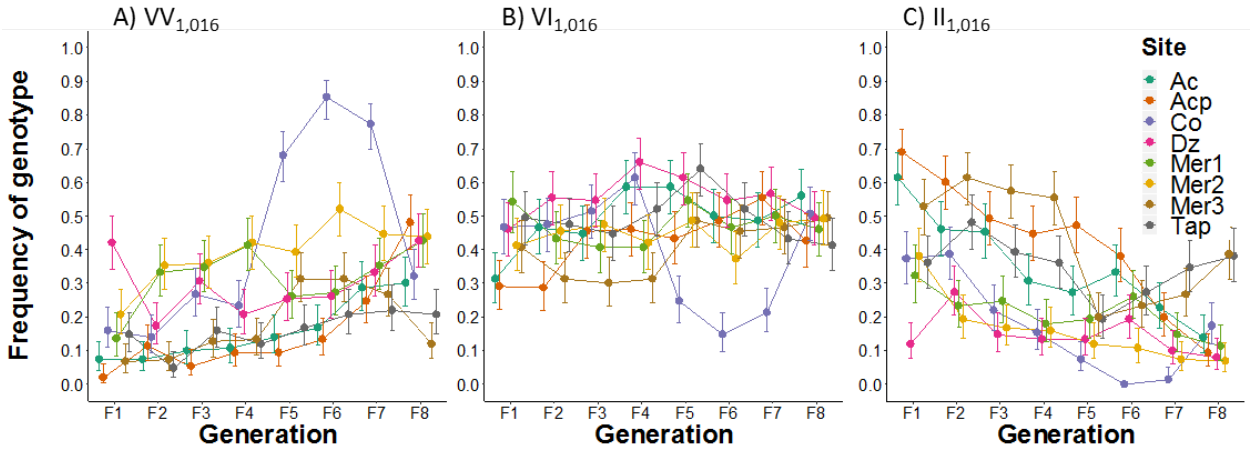


Figure 4.3. V1,016I genotypic frequencies over eight generations with the relaxation of pyrethroids.

Table 4.2. Pearson's correlation coefficient among collection sites V1,016I genotypic frequency and generations without exposure of pyrethroids.

	$VV_{1,016}$		$VI_{1,016}$		$II_{1,016}$	
Site	**Pearson r**	***P* value**	**Pearson r**	***P* value**	**Pearson r**	***P* value**
Ac	0.9301	0.0008	0.6406	0.0870	-0.9418	0.0005
Acp	0.8156	0.0136	0.7455	0.0338	-0.9619	0.0001
Co	0.6499	0.0811	-0.4268	0.2916	-0.7886	0.0200
Dz	0.2371	0.5718	0.1178	0.7811	-0.4836	0.2247
Mer1	0.5472	0.1604	0.0404	0.9244	-0.8005	0.0170
Mer2	0.8062	0.0156	0.3131	0.4502	-0.8753	0.0044
Mer3	0.5830	0.1293	0.7033	0.0516	-0.7085	0.0492
Tap	0.7559	0.0300	-0.1720	0.6838	-0.3615	0.3789
Across all	0.4497	0.0002	0.1462	0.2489	-0.5452	<0.0001

F1,534C in *Ae. aegypti* collections following the removal of pyrethroids. We genotyped F1,534C in each of the same mosquitoes for which V1,016I genotype frequencies had been determined. **Table 4.1S** lists the F1,534C genotypes counts, and **Table 4.2S** and **Figure 4.4A** shows C1,534 allele frequencies in all three replicates in different collections over eight generations following removal from pyrethroid exposure. C1,534 allele frequencies did not differ between replicates in 77% of the collections; 23% (15 cases) showed differences among replicates (**Table 4.2S and Figure 4.4A**). The F1,534C genotypes segregated in 152 out of 192 analyses. 148 populations were in HWE (**Table 4.2S**). The other four populations that were not in HWE had an excess of both homozygotes.

Figure 4.4B plots the average of C1,534 allele frequencies among all three replicates and displayed at the base of each graph the correlations and significance between C1,534 allele frequencies and the generation number. Across all collections, C1,534 allele frequencies decreased over time (r = -0.3024, p = 0.0152). Individually, in collection site Acp, Co, and Mer 2, C1,534 allele frequencies declined rapidly. At Mer1 there was actually a positive correlation while Mer3 and Tap had no changes in the C1,534 allele frequency across the eight generations.

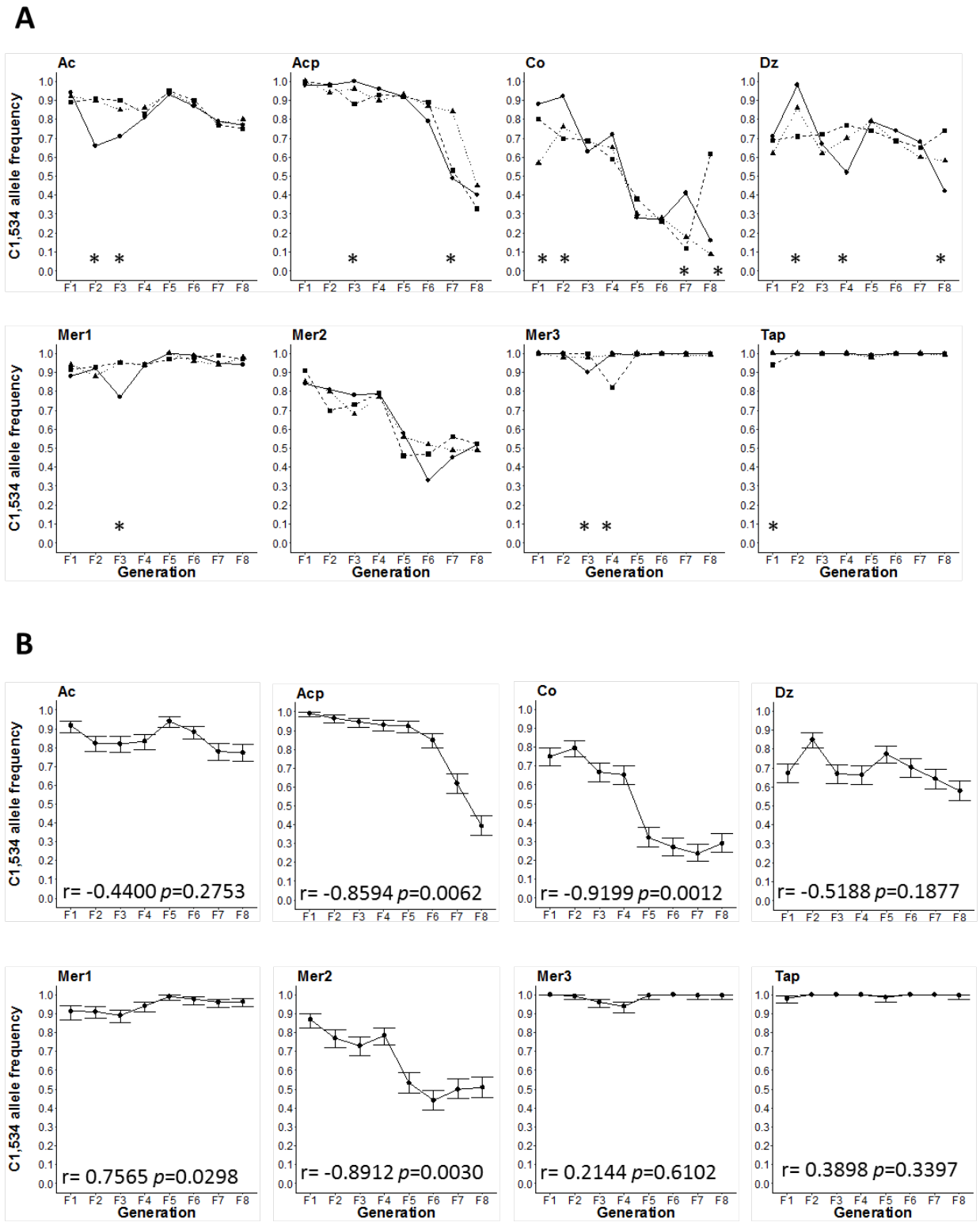


Figure 4.4. C1,534 allele frequency of *Ae. aegypti* of eight generation of pyrethroid removal in eight collection from Southern Mexico and Pearson's correlation coefficient and probability. A) All replicates shown, * indicates significant differences between replicates and B) Average allele frequencies, error bars represent 95% HDI.

Figure 4.5 displays F1,534C genotypic frequencies ($FF_{1,534}$, $FC_{1,534}$, $CC_{1,534}$) over eight generations. Correlations between genotype frequencies and generation are provided in **Table 4.3**. Five correlation coefficients for $FF_{1,534}$ were positive and two were significant (Co, Mer2). Five of the correlation coefficients for $FC_{1,534}$ were positive and two were significant (Acp, Mer2), and only Mer1 had a significant negative correlation. Five of the $CC_{1,534}$ were negative and three were significant (Acp, Co, Mer2), and only Mer1 had a significant positive correlation. Across all collections sites, there was a positive correlation (r = 0.3399, p=0.006) between $FF_{1,534}$ frequencies and generation number. The frequencies of $FC_{1,534}$ did not change over the eight generations (r = 0.2078, P=0.0994). As expected $CC_{1,534}$ genotypic frequencies decreased significantly over eight generations (r = -0.3024, P<0.0152).

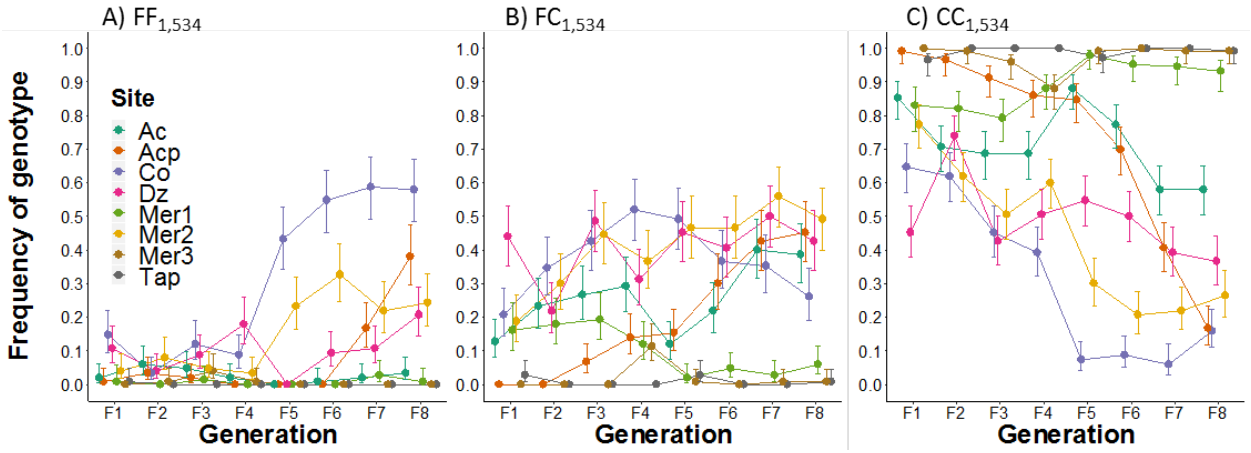


Figure 4.5. Genotypic frequencies of F1,534C over eight generations without exposure of pyrethroids.

Table 4.3. Pearson's correlation coefficient among collection sites F1,534C genotypic frequency and generations without exposure of pyrethroids.

	$FF_{1,534}$		$FC_{1,534}$		$CC_{1,534}$	
Site	**Pearson r**	***P* value**	**Pearson r**	***P* value**	**Pearson r**	***P* value**
Ac	-0.363	0.3759	0.6537	0.0788	-0.5460	0.1615
Acp	0.6952	0.0556	0.9711	<0.0001	-0.9110	0.0016
Co	0.8953	0.0026	0.0528	0.9012	-0.8996	0.0023
Dz	0.3782	0.3555	0.3733	0.3624	-0.5175	0.189
Mer1	0.2729	0.5131	-0.8254	0.0116	0.7953	0.0183
Mer2	0.7997	0.0172	0.8748	0.0045	-0.9131	0.0015
Mer3	-0.3423	0.4066	-0.0164	0.9692	0.1308	0.7575
Tap	-0.5774	0.1340	-0.2701	0.5177	0.3309	0.4233
Across all	0.3399	0.006	0.2078	0.0994	-0.3024	0.0152

Linkage disequilibrium. We performed pairwise linkage disequilibrium analyses between alleles in V1,016I and F1,534C in each generation at each collection site, for a total of 64 mosquito collections. **Table 4.4** shows the linkage disequilibrium coefficients (R_{ij}), χ^2 and the probability value obtained between pairwise loci. Alleles were segregated in 47 out of 64 mosquito collections. Mosquito collections $AcpF_1$, $TapF_2$ to $TapF_8$, $Mer1F_1$, $Mer1F_5$- F_6, $Mer3F_1$, and $Mer3F_4$-F_8 did not segregate. Mosquito collections where alleles segregated were in linkage disequilibrium with the exception of CoF_8. The R_{ij} values ranged between 0.15-0.85 among collections. **Figure 4.6** shows a strong positive correlation between the frequencies of the two resistant alleles at loci 1,016 and 1,534 (0.8474, p=<0.0001).

Table 4.4. ***Ae. aegypti's*** **linkage disequilibrium coefficients between loci 1,016 and 1,534 over eight generation without exposure to pyrethroids.**

		1,016-1,534					1,016-1,534		
Site	**Generation**	R_{ij}	χ^2	***Prob***	**Site**	**Generation**	R_{ij}	χ^2	***Prob***
Acp					**Dz**				
	F2	0.40262	24.32	0.0001		F1	0.47671	34.09	0.0001
	F3	0.40043	24.05	0.0001		F2	0.44321	29.46	0.0001
	F4	0.40285	24.34	0.0001		F3	0.50702	38.56	0.0001
	F5	0.53718	43.28	0.0001		F4	0.32897	16.23	0.0001
	F6	0.52471	41.3	0.0001		F5	0.35	18.38	0.0001
	F7	0.65434	64.22	0.0001		F6	0.55227	45.75	0.0001
	F8	0.85375	109.33	0.0001		F7	0.47286	33.54	0.0001
Tap						F8	0.52979	42.1	0.0001
	F1	0.18597	5.19	0.0227	**Co**				
Mer1						F1	0.63877	61.2	0.0001
	F2	0.3114	14.55	0.0001		F2	0.46501	32.43	0.0001
	F3	0.21116	6.69	0.0097		F3	0.61457	56.65	0.0001
	F4	0.30385	13.85	0.0002		F4	0.67762	68.88	0.0001
	F7	0.20948	6.58	0.0103		F5	0.67333	68.01	0.0001
	F8	0.23208	8.08	0.0045		F6	0.38327	22.03	0.0001
Mer2						F7	0.6995	73.39	0.0001
	F1	0.44441	29.63	0.0001		F8	0.15305	3.51	0.0609
	F2	0.47953	34.49	0.0001	**Ac**				
	F3	0.4159	25.95	0.0001		F1	0.54278	44.19	0.0001
	F4	0.30371	13.84	0.0002		F2	0.59628	53.33	0.0001
	F5	0.74874	84.09	0.0001		F3	0.68619	70.63	0.0001
	F6	0.7115	75.93	0.0001		F4	0.49335	36.51	0.0001
	F7	0.6068	55.23	0.0001		F5	0.30868	14.29	0.0002
	F8	0.66266	63.23	0.0001		F6	0.33043	16.38	0.0001
Mer3						F7	0.59792	53.63	0.0001
	F2	0.20207	6.12	0.0133		F8	0.4856	35.37	0.0001
	F3	0.42021	26.49	0.0001					

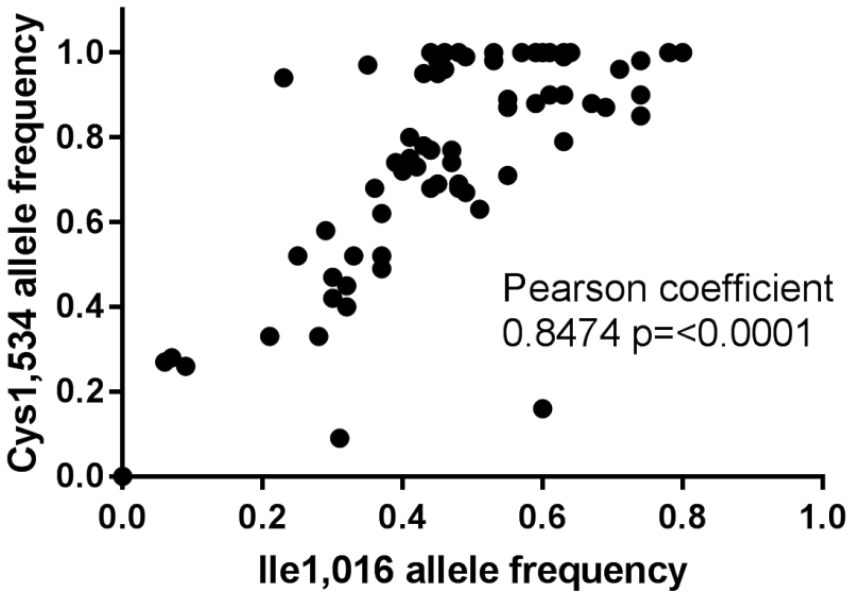


Figure 4.6. Correlation between resistant alleles at loci 1,016 and 1,534.

Figure 4.7 plotted the frequency of the four potential di-locus haplotypes over eight generations and **Table 4.5** displayed the correlation and the significance between haplotype frequencies and generation. The frequency of the susceptible $V_{1,016}/F_{1,534}$ haplotype increased over time ($r = 0.323$, $p = 0.009$) across all collections. Individually, collection site Acp, Co, and Mer2 had a significant increase in the susceptible haplotype over time (**Figure 4.7A** and **Table 4.5**). The frequency of $V_{1,016}/C_{1,534}$ haplotype increased in two collection sites, Ac and Mer1 (**Figure 4.7B** and **Table 4.5**). Interestingly, the frequencies of the $I_{1,016}/F_{1,534}$ haplotype remained low over time (**Figure 4.7C**) but reached as high as 0.28 in Co at the F_8 generation. The frequency of the resistant $I_{1,016}/C_{1,534}$ haplotype decreased over time ($r = -0.516$, $p =<0.0001$) across all collection sites. Individually, collection site Ac, Acp, Co, and Mer2 had a significant decreased in the resistant $I_{1,016}/C_{1,534}$ haplotype (**Figure 4.7D** and **Table 4.5**).

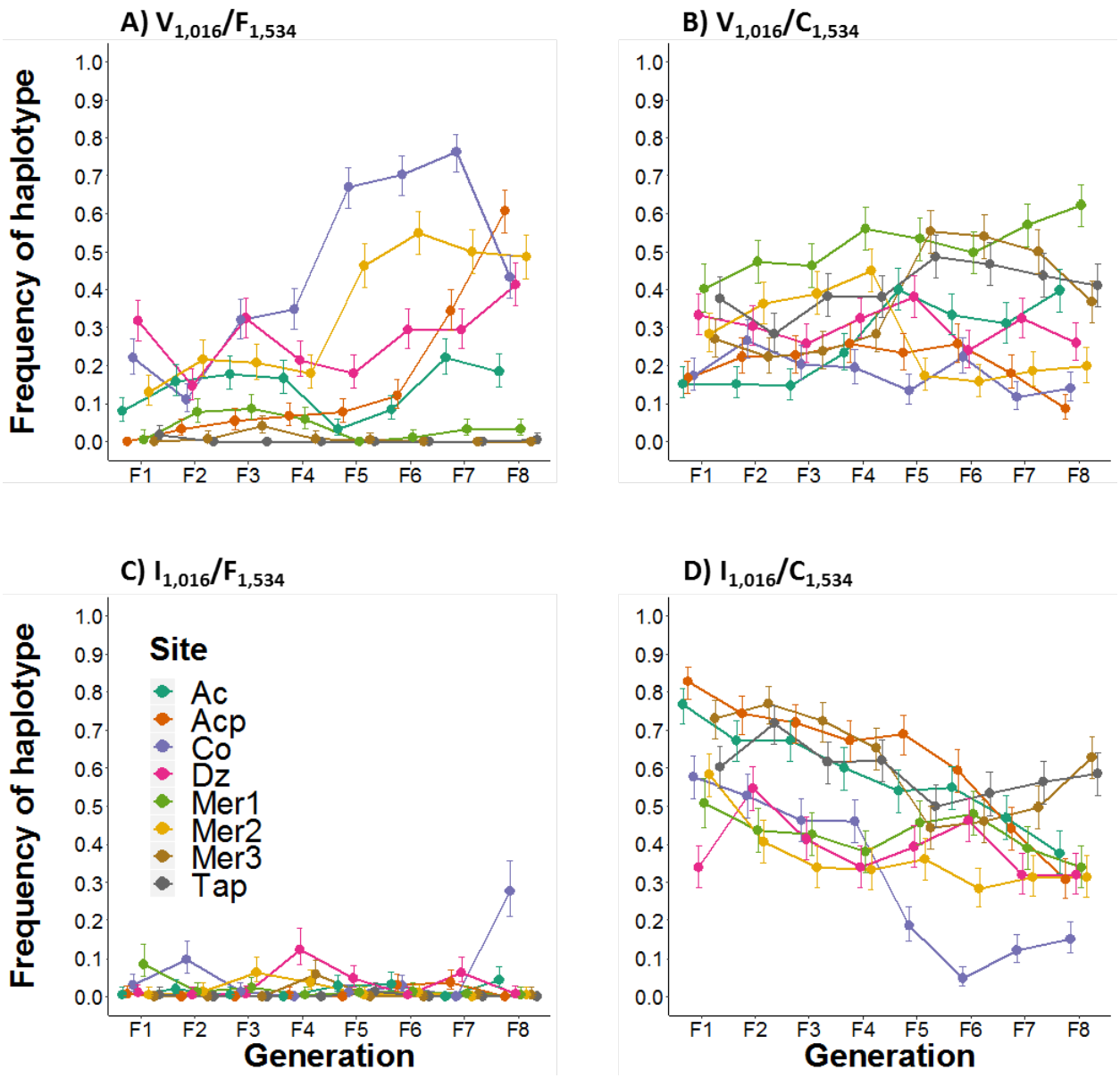


Figure 4. 7. Frequencies of the four potential haplotypes plotted by generation. A) Frequency of the susceptible $V_{1,016}/F_{1,534}$ haplotype, B) Frequency of the V1,016/C1,534 haplotype, C) Frequency of the $I_{1,016}/F_{1,534}$ haplotype, D) Frequency of the resistant $I_{1,016}/C_{1,534}$ haplotype.

Table 4. 5. Pearson's correlation and *p* value for four haplotypes at di-locus V1016I/F1534C.

	$V_{1,016}/F_{1,534}$		$V_{1,016}/C_{1,534}$		$I_{1,016}/F_{1,534}$		$I_{1,016}/C_{1,534}$	
Site	**Pearson r**	***P* value**	**Pearson r**	***P* value**	**Pearson r**	***P* value**	**Pearson r**	***P* value**
Ac	0.282	0.500	0.868	0.005	0.512	0.195	-0.978	<0.0001
Acp	0.842	0.009	-0.356	0.386	0.432	0.285	-0.937	0.001
Co	0.754	0.031	-0.566	0.143	0.401	0.325	-0.907	0.002
Dz	0.429	0.289	-0.251	0.549	0.130	0.759	-0.388	0.342
Mer1	-0.268	0.521	0.873	0.005	-0.672	0.068	-0.618	0.103
Mer2	0.882	0.004	-0.633	0.092	-0.339	0.412	-0.770	0.026
Mer3	-0.340	0.410	0.677	0.065	-0.031	0.942	-0.688	0.059
Tap	-0.480	0.229	0.624	0.099	-0.051	0.904	-0.560	0.149
Overall	0.323	0.009	0.140	0.271	0.091	0.474	-0.516	<0.0001

Temporal analysis of di-locus genotypes. Figure 4.8 shows the frequency of nine di-locus genotype combinations (3 genotypes at 2 loci) and **Table 4.6** lists the correlation between frequencies of each di-locus genotype and the generations without pyrethroid exposure. We found the nine genotype combinations in 9,563 mosquitoes analyzed. However, the di-locus coefficient among the nine graphs was only significant for the wild type susceptible $VV_{1,016}/FF_{1,534}$ and the dual resistant $II_{1,016}/CC_{1,534.}$ As expected the correlation of $VV_{1,016}/FF_{1,534}$ was a positive (r = 0.3376, $p = 0.0064$) indicating an increase while the correlation of $II_{1016}/CC_{1534.}$was a negative (r = -0.5465, $p < 0.0001$) indicating a decline.

Figure 4.8A shows that $VV_{1,016}/FF_{1,534}$ occurred at very low frequencies in the first four generations (maximum frequency = 0.125). By generation F_8, Acp and Mer 1 and 2 had significantly higher frequencies of $VV_{1,016}/FF_{1,534}$ as compared with F_1 and showed a strong correlation with generations (r = 0.7101 $p = 0.0484$ and r = 0.8243 $p= 0.0118$, respectively). Co had an increase in the double susceptible homozygote at generation F_5, F_6 and F_7 but decreased again at F_8 (**Figure 4.8A**).

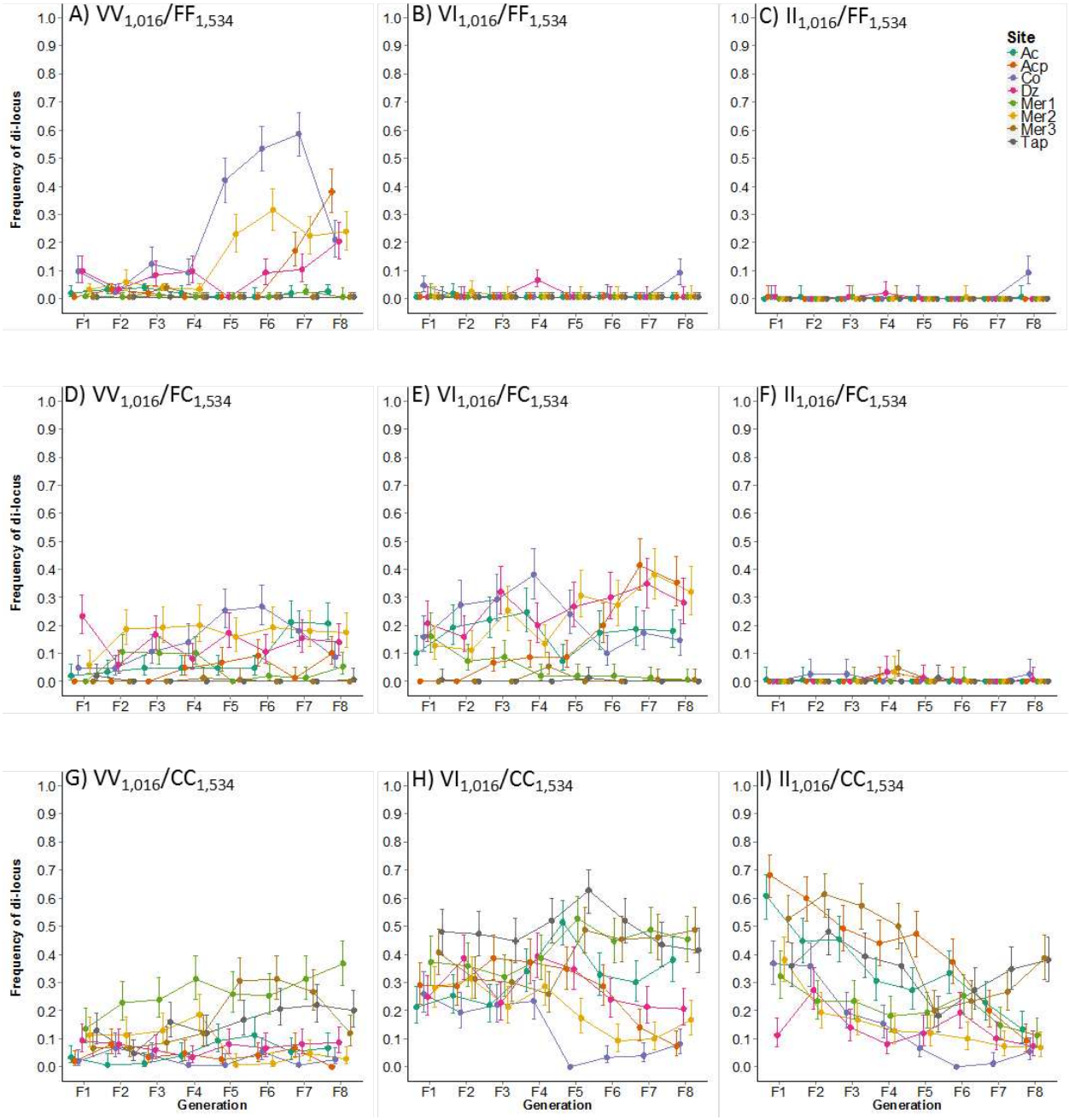


Figure 4. 8. Frequency of the nine di-locus genotypes over eight generations without pyrethroid exposure.

Table 4. 6. Person's correlations and p value for nine di-locus genotypes at loci V1,016I and F1,534C.

	$VV_{1,016}/FF_{1,534}$		$VI_{1,016}/FF_{1,534}$		$II_{1,016}/FF_{1,534}$	
Site	**Pearson r**	***P* value**	**Pearson r**	***P* value**	**Pearson r**	***P* value**
Ac	-0.3225	0.4360	-0.4124	0.31	0.1260	0.7663
Acp	0.7101	0.0484	-	-	-0.5774	0.1340
Co	0.6830	0.0619	0.3135	0.4496	0.5493	0.1585
Dz	0.5151	0.1915	-0.0825	0.8461	-0.3693	0.3679
Mer1	0.2980	0.4734	-0.5774	0.134	-0.2474	0.5546
Mer2	0.8243	0.0118	-0.4124	0.31	0.2474	0.5546
Mer3	-0.2531	0.5454	-	-	-0.0825	0.8461
Tap	-	-	-	-	-	-
Across all	0.3376	0.0064	0.0249	0.8451	0.1370	0.2803

	$VV_{1,016}/FC_{1,534}$		$VI_{1,016}/FC_{1,534}$		$II_{1,016}/FC_{1,534}$	
Site	**Pearson r**	***P* value**	**Pearson r**	***P* value**	**Pearson r**	***P* value**
Ac	0.8132	0.0141	0.1079	0.7993	-0.7559	0.0300
Acp	0.7280	0.0406	0.9162	0.0014	0.1260	0.7663
Co	0.5219	0.1846	-0.4137	0.3082	-0.0563	0.8946
Dz	-0.1429	0.7357	0.6513	0.0802	0.0724	0.8648
Mer1	-0.2719	0.5148	-0.8585	0.0064	0.0000	>0.9999
Mer2	0.4626	0.2485	0.8381	0.0094	-0.0825	0.8461
Mer3	-0.0357	0.9331	0.0494	0.9076	-0.0825	0.8461
Tap	-0.3711	0.3654	-0.2143	0.6103	0.0825	0.8461
Across all	0.2048	0.1046	0.1817	0.1507	-0.0471	0.712

	$VV_{1,016}/CC_{1,534}$		$VI_{1,016}/CC_{1,534}$		$II_{1,016}/CC_{1,534}$	
Site	**Pearson r**	***P* value**	**Pearson r**	***P* value**	**Pearson r**	***P* value**
Ac	0.6439	0.0849	0.5632	0.1461	-0.9390	0.0005
Acp	-0.2323	0.5799	-0.6772	0.0651	-0.9628	0.0001
Co	-0.2698	0.5182	-0.7866	0.0206	-0.9141	0.0015
Dz	0.0398	0.9255	-0.4257	0.293	-0.4131	0.3091
Mer1	0.8522	0.0072	0.7112	0.0479	-0.8105	0.0147
Mer2	-0.6506	0.0806	-0.8032	0.0164	-0.8622	0.0059
Mer3	0.6035	0.1132	0.6312	0.0933	-0.7211	0.0435
Tap	0.7993	0.0173	-0.1480	0.7266	-0.3517	0.3930
Across all	0.1757	0.165	-0.0899	0.48	-0.5465	<0.0001

Association between resistance allele frequencies, generation number and RR. Tables 4.3S and 4.4S show the LC_{50}s of permethrin and deltamethrin. Not all of the mosquito collections adjusted to the logistic regression model and there were not enough mosquitoes available to repeat the experiment. **Figure 4.9A** shows the positive correlation between permethrin RR and the frequency of I1,016 (in green) (r = 0.7080; $p \leq 0.0001$) and C1,534 (r = 0.7447; $p \leq 0.0001$) (in orange). **Figure 4.9B** shows the positive (in green) (r = 0.4557; $p = 0.0252$) correlation between deltamethrin RR and I1,016 frequency. The C1,534 allele frequency was not significantly correlated ($r = 0.3167$. $p = 0.1316$) with deltamethrin RR.

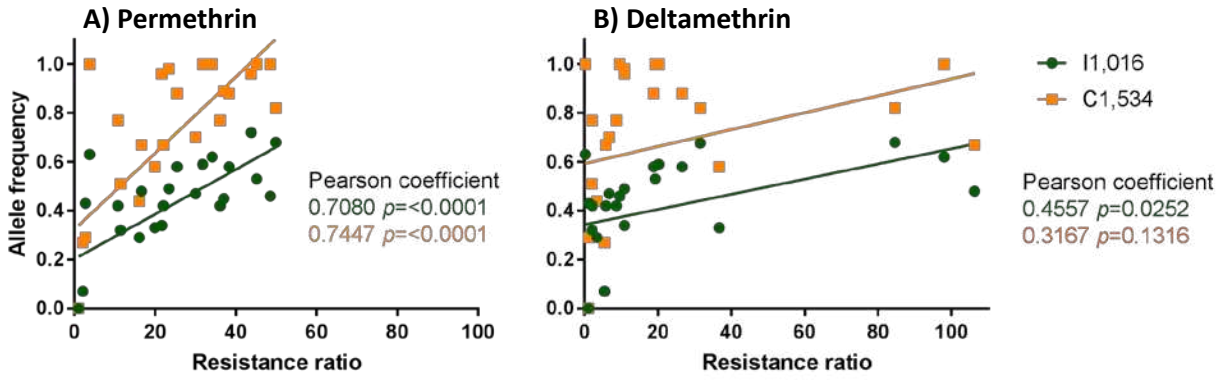


Figure 4.9. Correlation between the resistant alleles at loci 1,016 and C1,534 and the resistance ratio of permethrin (A) and deltamethrin (B).

Figure 4.10 shows the RR of permethrin and deltamethrin in the eight collections evaluated at generations F_3, F_6 and F_8. Permethrin RR (in red) ranged between 17 and 50-fold in generation F_3, between 2 and 49-fold in F_6 and between 3 and 36-fold in F_8. There was a significant decline in permethrin RR over generations F_3, F_6 and F_8 ($r = -0.4512$, $p = 0.0350$).

Figure 4.10 also shows the decline in RR of deltamethrin (in black) evaluated in the same generations as for permethrin susceptibility. The decline was more evident in the

deltamethrin RR (r = -0.6688, p = 0.0009) as compared with the permethrin RR. The deltamethrin RR ranged between 6 and 106-fold in generation F_3, by F_6 the RR ranged from 3 - 27-fold, and by F_8 from 0.18 - 37-fold.

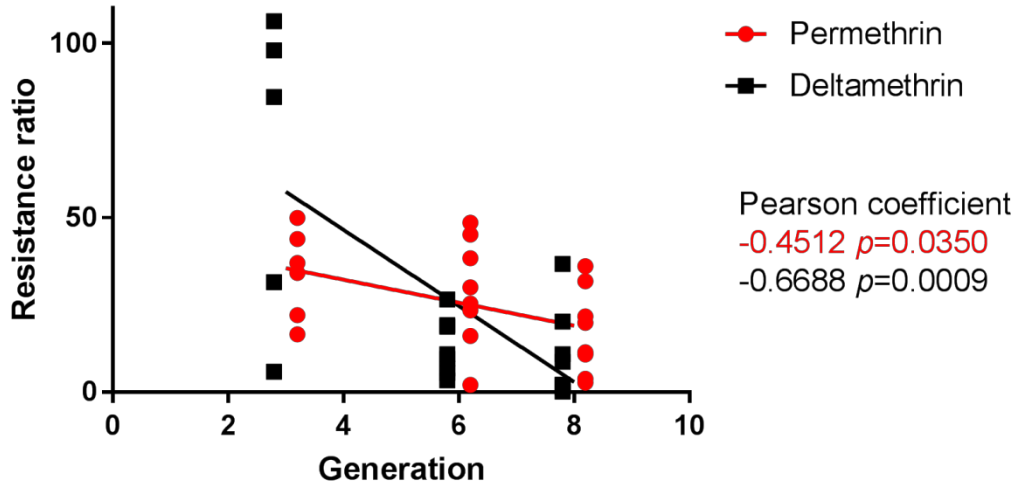


Figure 4. 10. Pearson's correlation between resistant ratio of permethrin and deltamethrin and generations without exposure of pyrethroids.

Association between resistance allele frequencies, RR, and fecundity. There was a negative correlation between the average fecundity measured in the eight collections over generations F_3, F_6 and F_8 and the frequencies of the resistance alleles (**Figure 4.11**). Fecundity tended to be lower in collections with higher frequencies of I1,016 and C1,534 but fecundity increased as the resistance frequencies decreased. There was a negative correlation between the average fecundity measured in the eight collections over generations F_3, F_6 and F_8 and RR (**Figure 4.12**). Fecundity tended to be lower in collections with higher RR but increased as the RR became smaller.

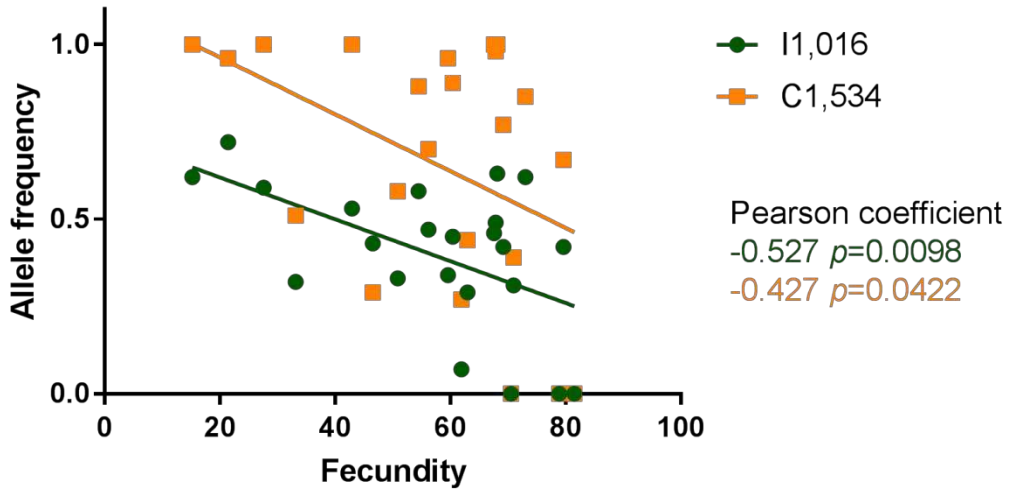


Figure 4. 11. Correlation of fecundity with resistant allele frequencies at loci 1,016 and 1,534.

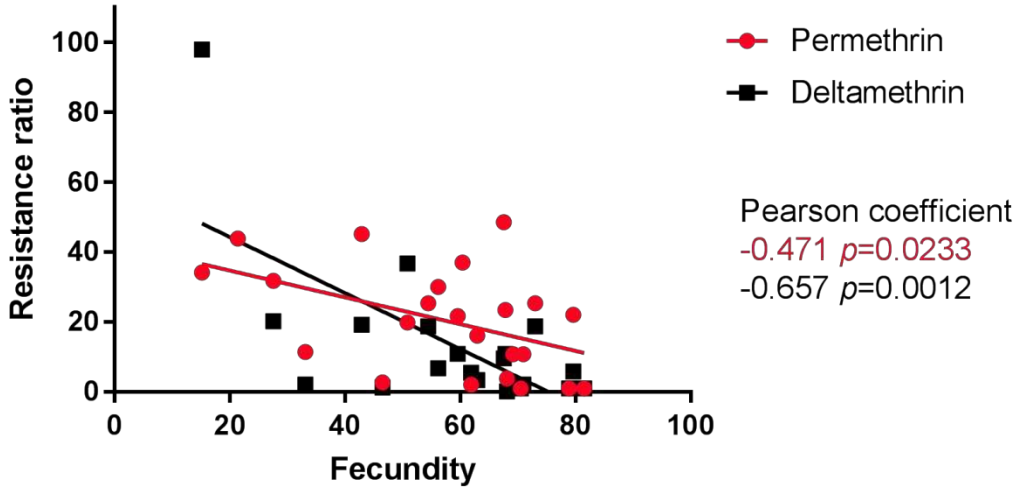


Figure 4. 12. Correlation of fecundity and resistant ratio for permethrin and deltamethrin.

Discussion

In this study we established eight colonies of *Aedes aegypti* from the field and maintained them in a pyrethroid free environment over eight generations. We demonstrated that the frequency of the *Ae. aegypti* pyrethroid resistance alleles I1,016 and C1,534 decline when pyrethroid pressure is removed in the laboratory (**Figures. 4.2** and **4.4**, **Tables 4.2** and **4.3**). However, the pattern of decline appears to be strain dependent with some having a steady rate of decline (Ac, Acp, Mer2 in **Figure 4.2**, Acp and Mer2 in **Figure 4.4**), some showing a shallow decline (e.g. Mer1, Tap in **Figure 4.2**; Ac, Mer2 in **Figure 4.4**) and others displaying no net change over eight generations (**Figure 4.2** Dz; **Figure 4.4**.Ac, Mer3, and Tap). A more surprising result was that in Co and Mer3, the frequencies of I1,016 actually increased following a precipitous drop. Likewise the frequencies of C1,534 in Mer1, Co, Mer2 actually increased in frequency after a drop.

There are a wide variety of possible causes for this heterogeneity in gene frequency trajectories among collections across 8 generations. It is unlikely that these shifts arose from founder's effects because we analyzed three replicates of 50 adult mosquitoes for each of the eight collection sites. Note also that the 95% HDI was very small in all graphs. Second, the initial frequencies of I1,016 varied from 0.65-1 (**Figure 4.2**) . This could influence the entire trajectory of I1,016 through the selection process with collections having a high frequency at F_1 also having a greater frequency in F_8. Collections having an intermediate frequency at F_1 would have a lower frequency in F_8.

Third, metabolic resistance may account for much of this heterogeneity. In a quantitative trait locus (QTL) mapping study, Saavedra-Rodriguez et al [73] reported that 58.6% of the variation in kdr could be accounted for by I1,016 but that a number of different QTL located

throughout the genome accounted for the remainder. Saavedra-Rodriguez et al (2012) [116] used the 'Aedes Detox' microarray [117] and showed an inverse relationship between I1,016 frequencies and the numbers of differentially transcribed metabolic genes.

Fourth, the low frequency of $VI_{1,016}/FF_{1,534}$, $II_{1,016}/FF_{1,534}$, and $II_{1,016}/FC_{1,534}$ noted in this and two previous studies [99, 113] may account for the increases in I1,016 frequency collections like those in Co and Mer3. For example, if I1,016 and F1,534 are in *cis* phase then selection for F1,534 after removal of pyrethroid pressure might cause increases in I1,016. This would arise from lack of recombination rather than through selection for I1,016. Conversely if I1,016 is *trans* with F1,534 then selection for F1,534 would not affect the frequency of I1,016. If V1,016 is *cis* with C1,534 then selection for V1,016 might increase C1,534 (e.g.Mer2, Co in **Figure 4.4**). At present we don't have any tools to identify the phase of alleles at these two loci. They are too far apart to be amplified by PCR or cloned to identify haplotypes.

We examined the relationship between fecundity, RR and resistant allele frequencies and found that individuals with higher frequencies of I1,016 and C1,534 tended to lay fewer eggs than susceptible individuals. A study in Brazil also showed a similar decline in fecundity of resistant field *Ae. aegypti* population [1]. Diniz et al (2015) [2] suggested that fecundity was compromised in temephos resistant populations of *Ae. aegypti* due to a metabolic resistance mechanism. We did not evaluate metabolic resistance. Further study of the levels of detoxifying enzymes is needed to understand how they impact the fitness of resistant populations.

Deltamethrin RRs appear to be correlated with I1,016 allele frequency but not with C1,534 allele frequencies, while permethrin RRs correlate with both allele frequencies. We speculate that there are other resistant mechanisms that could drive resistance to deltamethrin, such as detoxifying enzymes or other mutations in the voltage-gated sodium channel (vgsc) that

have not been identified. However, the resistance to permethrin appears to be driven heavily by both resistant alleles. The expression of C1,534 in *Xenopus* oocytes and exposure to both pyrethroids demonstrated that the resistant amino acid substitution in 1,534 is sensitive to permethrin but not for deltamethrin, which is consistent with our results [29, 96]. The resistant amino acid substitution I1,016, has been found in many resistant populations in the Americas [67, 87, 101, 118, 119] and it has been shown that it is in linkage with C1,534[99]. Interestingly, the I1,016 mutation was functionally expressed in *Xenopus* oocytes and did not show an alteration to the sodium channel sensitivity to both pyrethroids [29]. Our data indicate a positive correlation of I1,016 allele frequency with the RR of both pyrethroids. It is clear that more information is needed to understand the role that I1,016 plays in *Ae. aegypti* resistance.

F1,534C genotypes exhibited a different distribution and dynamic over time as compared with locus V1,016I. The decline of C1,534 allele frequency was reflected in the drop of the resistant homozygote $CC_{1,534}$ genotype frequency in mosquitoes from Acp, Co, and Mer2. The frequencies of F1,534C genotypes were very heterogeneous among collection sites. For instance, just one (Acp) of three collection sites (Acp, Tap and Mer3) with high initial frequencies of the resistant homozygote $CC_{1,534}$ exhibit a decline in their frequency over time while the other two collection sites (Tap and Mer3) remained high across generations. A possible explanation is that Tap and Mer3 had no susceptible or heterozygote genotypes to compete with the resistant genotype, the populations were fixed for C1,534.

Finally, these findings suggest that *kdr* I1,016 and C1,534 have a fitness cost and that fecundity is one of the biological traits that could be affected. Removing the insecticide and assuming a negative fitness associated with *kdr* mutations may allow the decline of resistant allele frequencies and result in the loss of pyrethroids resistance. However, the rate of loss of

resistance will vary among populations. In an operational mosquito control context, our findings indicate that the decline of resistant alleles is possible when pyrethroids are removed for at least 8 generations.

We want to clarify that this study indicates that the loss of pyrethroid resistance is unlikely to follow a smooth linear or exponential decline for many reasons, such as epistatic interactions between alleles. We do not know if the selection pressure applied in laboratory settings follows the same trend as selection pressures in the field. In addition, this study also indicates that the decline in resistance is not consistent among all collections. This variation highly depends on the genetic background of each population; some populations could take much longer to lose resistance while others may lose resistance much faster over time.

Chapter 5: Concluding Remarks

Based upon our observations on the evolution of three mutations in the vgsc that are associated with pyrethroid resistant *Ae. aegypti* populations in Mexico from 2000–2016 we conclude:

1) There are three mutations in the vgsc that are associated with pyrethroid resistant *Ae. aegypti* populations in Mexico. A valine at locus 410 (V410) confers susceptibility while leucine (L410) confers resistance. A valine at locus 1,016 (V1,016) confers susceptibility while isoleucine (I1,016) confers resistance. A phenylalanine at locus 1,534 (F1,534) confers susceptibility while cysteine (C1,534) confers resistance.
2) Pyrethroid resistance required the sequential evolution of mutations. C1,534 is likely to have occurred first and probably enabled the I1,016 mutation to survive.
3) V410L was initially detected in a pyrethroid resistant insectary strain from Brazilian *Ae. aegypti* populations. We screened V410L in 25 *Ae. aegypti* historical collections from Mexico. The first heterozygote appeared in 2002 and frequencies have increased in the last 16 years, alongside I1,016 and C1,534.
4) L410 showed a strong association between 1,534 and 1,016 mutations. Individuals with the triple homozygote resistant genotype had higher survival rates after pyrethroid exposure.
5) Interestingly, electrophysiology studies have shown that only five mutations are functionally confirmed to reduce vgsc sensitivity to pyrethroids. These are P989, M1,011, G1,016, C1,534 and most recently L410. I1,016 has not been shown to reduce vgsc sensitivity. How I1,016 has coevolved with V410L and C1,534and requires further research.

6) A key component of resistance management assumes that there will be a negative fitness associated with resistance alleles so that when insecticides are removed, resistance alleles will decline in frequency. The frequency of the *Ae. aegypti* pyrethroid resistance alleles I1,016 and C1,534 declined when pyrethroid pressure was removed in the laboratory. However, the pattern of decline was strongly strain dependent.
7) In agreement with earlier studies, fecundity was negatively correlated with the frequency of resistance alleles. This suggests that there is a fitness cost to resistance with the alleles studied.
8) Because most vgsc alleles are recessive, there is no way that the initial susceptibility of populations (before the introduction of a pyrethroid) will ever be fully recovered. This is because recessive alleles will be masked by the dominant susceptible vgsc alleles.

References

1. Brito, L.P., Linss, J.G., Lima-Camara, T.N., Belinato, T.A., Peixoto, A.A., Lima, J.B., Valle, D., and Martins, A.J. (2013). Assessing the effects of Aedes aegypti kdr mutations on pyrethroid resistance and its fitness cost. PLoS One *8*, e60878.
2. Diniz, D.F., de Melo-Santos, M.A., Santos, E.M., Beserra, E.B., Helvecio, E., de Carvalho-Leandro, D., dos Santos, B.S., de Menezes Lima, V.L., and Ayres, C.F. (2015). Fitness cost in field and laboratory Aedes aegypti populations associated with resistance to the insecticide temephos. Parasit Vectors *8*, 662.
3. Taylor, L.H., Latham, S.M., and Woolhouse, M.E. (2001). Risk factors for human disease emergence. Philos Trans R Soc Lond B Biol Sci *356*, 983-989.
4. Jones, Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L., and Daszak, P. (2008). Global trends in emerging infectious diseases. Nature *451*, 990-993.
5. McNeill, J.R. (2004). Yellow Jack and Geopolitics: Environment, Epidemics, and the Struggles for Empire in the American Tropics, 1650–1825. OAH Magazine of History *18*, 9-13.
6. Brown, J.E., Evans, B.R., Zheng, W., Obas, V., Barrera-Martinez, L., Egizi, A., Zhao, H., Caccone, A., and Powell, J.R. (2014). Human impacts have shaped historical and recent evolution in Aedes aegypti, the dengue and yellow fever mosquito. Evolution *68*, 514-525.
7. Brown, J.E., McBride, C.S., Johnson, P., Ritchie, S., Paupy, C., Bossin, H., Lutomiah, J., Fernandez-Salas, I., Ponlawat, A., Cornel, A.J., et al. (2011). Worldwide patterns of genetic differentiation imply multiple 'domestications' of Aedes aegypti, a major vector of human diseases. Proc Biol Sci *278*, 2446-2454.
8. Carter, H.R. (1931). Yellow Fever: An Epidemiological and Historical Study of Its Place of Origin., (Baltimore: Williams & Wilkins).
9. Pan American Health Organization, and Wold Health Organization (2017). Epidemiological update: Yellow fever. 16 March Edition. (Washington, D.C.: Pan American Health Organization/Wold Health Organization).
10. Bhatt, S., Gething, P.W., Brady, O.J., Messina, J.P., Farlow, A.W., Moyes, C.L., Drake, J.M., Brownstein, J.S., Hoen, A.G., Sankoh, O., et al. (2013). The global distribution and burden of dengue. Nature *496*, 504-507.
11. Pan American Health Organization, and World Health Organization (2013-2017). Number of reported cases of CHIK fever in the Americas by country or territory 2013-2014, 2015, 2016, 2017. (Washington, D.C.).
12. Campos, G.S., Bandeira, A.C., and Sardi, S.I. (2015). Zika Virus Outbreak, Bahia, Brazil. Emerg Infect Dis *21*, 1885-1886.
13. Pan American Health Organization, and World Health Organization (2018). Zika suspected and confirmed cases reported by countries and territoeires in the Americas Cumulative cases, 2015-2017. (Washington, D.C.: PAHO/WHO).
14. Foy, B.D., Kobylinski, K.C., Chilson Foy, J.L., Blitvich, B.J., Travassos da Rosa, A., Haddow, A.D., Lanciotti, R.S., and Tesh, R.B. (2011). Probable non-vector-borne transmission of Zika virus, Colorado, USA. Emerg Infect Dis *17*, 880-882.

15. Center for Disease Control and Prevention Cumulative Zika Virus Disease Case Counts in the United States, 2015-2018. April 4, 2018 Edition.
16. Dickson, L.B., Sanchez-Vargas, I., Sylla, M., Fleming, K., and Black, W.C.t. (2014). Vector competence in West African Aedes aegypti Is Flavivirus species and genotype dependent. PLoS Negl Trop Dis *8*, e3153.
17. Powell, J.R., and Tabachnick, W.J. (2013). History of domestication and spread of Aedes aegypti--a review. Mem Inst Oswaldo Cruz *108 Suppl 1*, 11-17.
18. Tabachnick, W.J. (1991). Evolutionary genetics and insect borne disease. The yellow fever mosquito, *Aedes aegypti*. American Entomologist *37*, 14-24.
19. McClelland, G.A.H. (1974). A worldwide survey of variation in scale pattern of the abdominal tergum of *Aedes aegypti* (L.) (Dipera: Culicidae). Trans. R. ent. Soc. Land. *126*, 239-259.
20. Tabachnick, W.J., Munstermann, L.E., and Powell, J.R. (1979). Genetic Distinctness of Sympatric Forms of Aedes Aegypti in East Africa. Evolution *33*, 287-295.
21. Pan American Health Organization, and World Health Organization (2016). Key messages for individuals and families regarding surveillance and control of *Aedes aegypti*: transmitter of dengue, chikungunya, Zika and other arbovirus diseases in the Americas.
22. Ayllon, T., Camara, D.C.P., Morone, F.C., Goncalves, L.D.S., Saito Monteiro de Barros, F., Brasil, P., Carvalho, M.S., and Honorio, N.A. (2018). Dispersion and oviposition of Aedes albopictus in a Brazilian slum: Initial evidence of Asian tiger mosquito domiciliation in urban environments. PLoS One *13*, e0195014.
23. Yu, S.J. (2015). The toxicology and biochemistry of Insecticides, 2nd Edition, (Boca Raton, FL: CRC Press).
24. Wang, S.Y., and Wang, G.K. (2003). Voltage-gated sodium channels as primary targets of diverse lipid-soluble neurotoxins. Cell Signal *15*, 151-159.
25. Klowden, M.J. (2013). Physiological systems in insects, Thrid Edition, (Elsevier/Academic Press).
26. Soderlund, D.M. (2010). State-Dependent Modification of Voltage-Gated Sodium Channels by Pyrethroids. Pestic Biochem Physiol *97*, 78-86.
27. Catterall, W.A., Goldin, A.L., and Waxman, S.G. (2005). International Union of Pharmacology. XLVII. Nomenclature and structure-function relationships of voltage-gated sodium channels. Pharmacol Rev *57*, 397-409.
28. Dudley, S.C., Jr., Chang, N., Hall, J., Lipkind, G., Fozzard, H.A., and French, R.J. (2000). mu-conotoxin GIIIA interactions with the voltage-gated Na(+) channel predict a clockwise arrangement of the domains. J Gen Physiol *116*, 679-690.
29. Du., Nomura, Y., Satar, G., Hu, Z., Nauen, R., He, S.Y., Zhorov, B.S., and Dong, K. (2013). Molecular evidence for dual pyrethroid-receptor sites on a mosquito sodium channel. Proc Natl Acad Sci U S A *110*, 11785-11790.
30. Dong, K., Du, Y., Rinkevich, F., Nomura, Y., Xu, P., Wang, L., Silver, K., and Zhorov, B.S. (2014). Molecular biology of insect sodium channels and pyrethroid resistance. Insect Biochem Mol Biol *50*, 1-17.
31. Vijverberg, H.P., van der Zalm, J.M., and van der Bercken, J. (1982). Similar mode of action of pyrethroids and DDT on sodium channel gating in myelinated nerves. Nature *295*, 601-603.

32. Davies, T.G., Field, L.M., Usherwood, P.N., and Williamson, M.S. (2007). DDT, pyrethrins, pyrethroids and insect sodium channels. IUBMB Life *59*, 151-162.
33. Zhorov, B.S., and Dong, K. (2017). Elucidation of pyrethroid and DDT receptor sites in the voltage-gated sodium channel. Neurotoxicology *60*, 171-177.
34. Silver, K.S., Song, W., Nomura, Y., Salgado, V.L., and Dong, K. (2010). Mechanism of action of sodium channel blocker insecticides (SCBIs) on insect sodium channels. Pestic Biochem Physiol *97*, 87-92.
35. Du, Nomura, Y., Zhorov, B.S., and Dong, K. (2016). Evidence for Dual Binding Sites for 1,1,1-Trichloro-2,2-bis(p-chlorophenyl)ethane (DDT) in Insect Sodium Channels. J Biol Chem *291*, 4638-4648.
36. Narahashi, T. (1986). Toxins that modulate the sodium channel gating mechanism. Ann. N. Y. Acad. Sci. *479*, 19.
37. Agengy., U.S.E.P. (2018). Pyrethrins and Pyrethroids.
38. Sparks, T.C. (2013). Insecticide discovery: an evaluation and analysis. Pestic Biochem Physiol *107*, 8-17.
39. Centro Nacional de Programas Preventivos y Control de Enfermedades (2018). Productos recomendados por el CENAPRECE para el combate de insectos vectores de enfermedades a partir de 2018. S.d.p.y.p.d.l. salud, ed.
40. Gammon, D.W., Brown, M.A., and Casida, J.E. (1981). Two classes of pyrethroid action in the cockroach. Pesticide Biochemistry and Physiology *15*.
41. Verschoyle, R.D., and Aldridge, W.N. (1980). Structure-activity relationships of some pyrethroids in rats. Archives of toxicology *45*.
42. Lawrence, L.J., and Casida, J.E. (1982). Pyrethroid toxicology: Mouse intracerebral structure - toxicity relationships. Pesticide Biochemistry and Physiology *18*.
43. Breckenridge, C.B., Holden, L., Sturgess, N., Weiner, M., Sheets, L., Sargent, D., Soderlund, D.M., Choi, J.S., Symington, S., Clark, J.M., et al. (2009). Evidence for a separate mechanism of toxicity for the Type I and the Type II pyrethroid insecticides. Neurotoxicology *30 Suppl 1*, S17-31.
44. O'Reilly, A.O., Khambay, B.P., Williamson, M.S., Field, L.M., Wallace, B.A., and Davies, T.G. (2006). Modelling insecticide-binding sites in the voltage-gated sodium channel. Biochem J *396*, 255-263.
45. Becker, N., Petric, D., Zgomba, M., Boase, C., Madon, M., Dahl, C., and Kaiser, A. (2010). Mosquitoes and their control, Second Edition, (Berlin: Springer).
46. Martins, A.J., Belinato, T.A., Lima, J.B., and Valle, D. (2008). Chitin synthesis inhibitor effect on Aedes aegypti populations susceptible and resistant to organophosphate temephos. Pest Manag Sci *64*, 676-680.
47. Committee, I.R.A. (2018). IRAC Mode of action classification scheme.
48. Nayar, J.K., and Ali, A. (2003). A review of monomolecular surface films as larvicides and pupicides of mosquitoes. J Vector Ecol *28*, 190-199.
49. Nelder, M., Kesavaraju, B., Farajollahi, A., Healy, S., Unlu, I., Crepeau, T., Ragavendran, A., Fonseca, D., and Gaugler, R. (2010). Suppressing Aedes albopictus, an emerging vector of dengue and chikungunya viruses, by a novel combination of a monomolecular film and an insect-growth regulator. Am J Trop Med Hyg *82*, 831-837.
50. Jayapriya, G., and Shoba, F.G. (2014). Evaluation of *Gambusia affinis* and *Bacillus thuringiensis* var. *israelensis* as *Culex quinquefasciatus* control agents. Journal of entomology and zoology studies *2*, 5.

51. Howard, A.F., Zhou, G., and Omlin, F.X. (2007). Malaria mosquito control using edible fish in western Kenya: preliminary findings of a controlled study. BMC Public Health *7*, 199.
52. Nyamah, M.A., Sulaiman, S., and Omar, B. (2011). Field observation on the efficacy of Toxorhynchites splendens (Wiedemann) as a biocontrol agent against Aedes albopictus (Skuse) larvae in a cemetery. Trop Biomed *28*, 312-319.
53. Schreiber, E.T. (2007). Toxorhynchites. J Am Mosq Control Assoc *23*, 129-132.
54. Kay, B.H., Tuyet Hanh, T.T., Le, N.H., Quy, T.M., Nam, V.S., Hang, P.V., Yen, N.T., Hill, P.S., Vos, T., and Ryan, P.A. (2010). Sustainability and cost of a community-based strategy against Aedes aegypti in northern and central Vietnam. Am J Trop Med Hyg *82*, 822-830.
55. Marten, G.G., and Reid, J.W. (2007). Cyclopoid copepods. J Am Mosq Control Assoc *23*, 65-92.
56. Tietze, N.S., Hester, P.G., Hallmon, C.F., Olson, M.A., and Shaffer, K.R. (1991). Acute toxicity of mosquitocidal compounds to young mosquitofish, Gambusia affinis. J Am Mosq Control Assoc *7*, 290-293.
57. Azevedo-Santos, V.M., Vitule, J.R., Garcia-Berthou, E., Pelicice, F.M., and Simberloff, D. (2016). Misguided strategy for mosquito control. Science *351*, 675.
58. Bay, E.C. (1967). Mosquito control by fish: a present-day appraisal. WHO Chron *21*, 415-423.
59. Federici, B.A. (1999). *Bacillus thuringeiensis* in Biological Control. In Handbook of biological control: principles and applications of biological control, 1st Edition, T.S. Bellows, T.W. Fisher, L.E. Caltagirone, D.L. Dahlsten, G. Gordh and C.B. Huffaker, eds. (San Diego, California: Academic Press).
60. Araujo, A.P., Araujo Diniz, D.F., Helvecio, E., de Barros, R.A., de Oliveira, C.M., Ayres, C.F., de Melo-Santos, M.A., Regis, L.N., and Silva-Filha, M.H. (2013). The susceptibility of Aedes aegypti populations displaying temephos resistance to Bacillus thuringiensis israelensis: a basis for management. Parasit Vectors *6*, 297.
61. Muspratt, J. (1963). Destruction of the Larvae of Anopheles Gambiae Giles by a Coelomomyces Fungus. Bull World Health Organ *29*, 81-86.
62. Maldonado-Blanco, M.G., Leal-Lopez, E.Y., Ochoa-Salazar, O.A., Elias-Santos, M., Galan-Wong, L.J., and Quiroz-Martinez, H. (2011). Effects of culture medium and formulation on the larvicidal activity of the mosquito pathogen Lagenidium giganteum (Oomycetes: Lagenidiales) against Aedes aegypti. Acta Trop *117*, 114-118.
63. Darbro, J.M., Johnson, P.H., Thomas, M.B., Ritchie, S.A., Kay, B.H., and Ryan, P.A. (2012). Effects of Beauveria bassiana on survival, blood-feeding success, and fecundity of Aedes aegypti in laboratory and semi-field conditions. Am J Trop Med Hyg *86*, 656-664.
64. Paula, A.R., Carolino, A.T., Silva, C.P., Pereira, C.R., and Samuels, R.I. (2013). Testing fungus impregnated cloths for the control of adult Aedes aegypti under natural conditions. Parasit Vectors *6*, 256.
65. Paula, A.R., Silva, L.E.I., Ribeiro, A., Butt, T.M., Silva, C.P., and Samuels, R.I. (2018). Improving the delivery and efficiency of fungus-impregnated cloths for control of adult Aedes aegypti using a synthetic attractive lure. Parasit Vectors *11*, 285.

66. Dantes, H.G., Farfan-Ale, J.A., and Sarti, E. (2014). Epidemiological trends of dengue disease in Mexico (2000-2011): a systematic literature search and analysis. PLoS Negl Trop Dis *8*, e3158.
67. Garcia, G.P., Flores, A.E., Fernandez-Salas, I., Saavedra-Rodriguez, K., Reyes-Solis, G., Lozano-Fuentes, S., Guillermo Bond, J., Casas-Martinez, M., Ramsey, J.M., Garcia-Rejon, J., et al. (2009). Recent rapid rise of a permethrin knock down resistance allele in Aedes aegypti in Mexico. PLoS Negl Trop Dis *3*, e531.
68. Harris, A.F., Rajatileka, S., and Ranson, H. (2010). Pyrethroid resistance in Aedes aegypti from Grand Cayman. Am J Trop Med Hyg *83*, 277-284.
69. Yanola, J., Somboon, P., Walton, C., Nachaiwieng, W., Somwang, P., and Prapanthadara, L.A. (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 Health *16*, 501-509.
70. Alvarez, L.C., Ponce, G., Saavedra-Rodriguez, K., Lopez, B., and Flores, A.E. (2015). Frequency of V1016I and F1534C mutations in the voltage-gated sodium channel gene in Aedes aegypti in Venezuela. Pest Manag Sci *71*, 863-869.
71. Wang, L., Nomura, Y., Du, Y., Liu, N., Zhorov, B.S., and Dong, K. (2015). A mutation in the intracellular loop III/IV of mosquito sodium channel synergizes the effect of mutations in helix IIS6 on pyrethroid resistance. Mol Pharmacol *87*, 421-429.
72. Black, W.C., and DuTeau, N.M. (1997). RAPD-PCR and SSCP analysis for insect population genetic studies. In The Molecular Biology of Insect Disease Vectors: A Methods Manual., Crampton J, Beard C and L. C, eds. (New York:: Chapman and Hall), pp. 361-373.
73. Saavedra-Rodriguez, K., Strode, C., Flores Suarez, A., Fernandez Salas, I., Ranson, H., Hemingway, J., and Black, W.C.t. (2008). Quantitative trait loci mapping of genome regions controlling permethrin resistance in the mosquito Aedes aegypti. Genetics *180*, 1137-1152.
74. Saavedra-Rodriguez, K., Urdaneta-Marquez, L., Rajatileka, S., Moulton, M., Flores, A.E., Fernandez-Salas, I., Bisset, J., Rodriguez, M., McCall, P.J., Donnelly, M.J., et al. (2007). A mutation in the voltage-gated sodium channel gene associated with pyrethroid resistance in Latin American Aedes aegypti. Insect Mol Biol *16*, 785-798.
75. Urdaneta-Marquez, L., Bosio, C., Herrera, F., Rubio-Palis, Y., Salasek, M., and Black, W.C.t. (2008). Genetic relationships among Aedes aegypti collections in Venezuela as determined by mitochondrial DNA variation and nuclear single nucleotide polymorphisms. Am J Trop Med Hyg *78*, 479-491.
76. Black, W.C., and Krafsur, E.S. (1985). A FORTRAN program for the calculation and analysis of two-locus linkage disequilibrium coefficients. Theor Appl Genet *70*, 491-496.
77. Cockerham, C.C., and Weir, B.S. (1977). Digenic Descent Measures for Finite Populations. Genetics Research *30*, 121-147.
78. Weir, B.S. (1979). Inferences About Linkage Disequilibrium. Biometrics *35*, 235-254.
79. Lunn, D.J., Thomas, A., Best, N., and Spiegelhalter, D. (2000). WinBUGS - A Bayesian modelling framework: Concepts, structure, and extensibility. Stat Comput *10*, 325-337.
80. Jones, C.M., Liyanapathirana, M., Agossa, F.R., Weetman, D., Ranson, H., Donnelly, M.J., and Wilding, C.S. (2012). Footprints of positive selection associated with a mutation (N1575Y) in the voltage-gated sodium channel of Anopheles gambiae. P Natl Acad Sci USA *109*, 6614-6619.

81. Pittendrigh, B., Reenan, R., ffrenchConstant, R.H., and Ganetzky, B. (1997). Point mutations in the Drosophila sodium channel gene para associated with resistance to DDT and pyrethroid insecticides. Mol Gen Genet *256*, 602-610.
82. He, H.Q., Chen, A.C., Davey, R.B., Ivie, G.W., and George, J.E. (1999). Identification of a point mutation in the para-type sodium channel gene from a pyrethroid-resistant cattle tick. Biochem Bioph Res Co *261*, 558-561.
83. Wang, S.Y., Barile, M., and Wang, G.K. (2001). A phenylalanine residue at segment D3-S6 in Nav1.4 voltage-gated Na+ channels is critical for pyrethroid action. Molecular Pharmacology *60*, 620-628.
84. Soderlund, D.M., and Knipple, D.C. (2003). The molecular biology of knockdown resistance to pyrethroid insecticides. Insect Biochem Molec *33*, 563-577.
85. Davies, T.G., and Williamson, M.S. (2009). Interactions of pyrethroids with the voltage-gated sodium channel. Bayer CropScience Journal. *62*.
86. Barbosa, S., Black, W.C.t., and Hastings, I. (2011). Challenges in estimating insecticide selection pressures from mosquito field data. PLoS Negl Trop Dis *5*, e1387.
87. Aponte, H.A., Penilla, R.P., Dzul-Manzanilla, F., Che-Mendoza, A., Lopez, A.D., Solis, F., Manrique-Saide, P., Ranson, H., Lenhart, A., McCall, P.J., et al. (2013). The pyrethroid resistance status and mechanisms in Aedes aegypti from the Guerrero state, Mexico. Pesticide Biochemistry and Physiology *107*, 226-234.
88. da-Cunha, M.P., Lima, J.B.P., Brogdon, W.G., Moya, G.E., and Valle, D. (2005). Monitoring of resistance to the pyrethroid cypermethrin in Brazilian Aedes aegypti (Diptera : Culicidae) populations collected between 2001 and 2003. Mem I Oswaldo Cruz *100*, 441-444.
89. Flores, A.E., Ponce, G., Silva, B.G., Gutierrez, S.M., Bobadilla, C., Lopez, B., Mercado, R., and Black, W.C. (2013). Wide Spread Cross Resistance to Pyrethroids in Aedes aegypti (Diptera: Culicidae) From Veracruz State Mexico. Journal of Economic Entomology *106*, 959-969.
90. Marcombe, S., Mathieu, R.B., Pocquet, N., Riaz, M.A., Poupardin, R., Selior, S., Darriet, F., Reynaud, S., Yebakima, A., Corbel, V., et al. (2012). Insecticide Resistance in the Dengue Vector Aedes aegypti from Martinique: Distribution, Mechanisms and Relations with Environmental Factors. Plos One *7*.
91. Soderlund, D.M., and Bloomquist, J.R. (1989). Neurotoxic Actions of Pyrethroid Insecticides. Annual Review of Entomology *34*, 77-96.
92. Brengues, C., Hawkes, N.J., Chandre, F., McCarroll, L., Duchon, S., Guillet, P., Manguin, S., Morgan, J.C., and Hemingway, J. (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.
93. Chang, C., Shen, W.K., Wang, T.T., Lin, Y.H., Hsu, E.L., and Dai, S.M. (2009). A novel amino acid substitution in a voltage-gated sodium channel is associated with knockdown resistance to permethrin in Aedes aegypti. Insect Biochem Molec *39*, 272-278.
94. Srisawat, R., Komalamisra, N., Eshita, Y., Zheng, M.Q., Ono, K., Itoh, T.Q., Matsumoto, A., Petmitr, S., and Rongsriyam, Y. (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.

95. Kushwah, R.B.S., Mallick, P.K., Ravikumar, H., Dev, V., Kapoor, N., Adak, T., and Singh, O.P. (2015). Status of DDT and pyrethroid resistance in Indian Aedes albopictus and absence of knockdown resistance (kdr) mutation. J Vector Dis *52*, 95-98.
96. Haddi, K., Tome, H.V.V., Du, Y., Valbon, W.R., Nomura, Y., Martins, G.F., Dong, K., and Oliveira, E.E. (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*, 46549.
97. Du, Nomura, Y., Zhorov, B.S., and Dong, K. (2016). Sodium Channel Mutations and Pyrethroid Resistance in Aedes aegypti. Insects *7*.
98. Du, Y.Z., Nomura, Y., Satar, G., Hu, Z.N., Nauen, R., He, S.Y., Zhorov, B.S., and Dong, K. (2014). Molecular evidence for dual pyrethroid-receptor sites on a mosquito sodium channel. Abstr Pap Am Chem S *248*.
99. Vera-Maloof, F.Z., Saavedra-Rodriguez, K., Elizondo-Quiroga, A.E., Lozano-Fuentes, S., and Black, W.C. (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*, e0004263.
100. Lima, E.P., Paiva, M.H.S., de Araujo, A.P., da Silva, E.V.G., da Silva, U.M., de Oliveira, L.N., Santana, A.E.G., Barbosa, C.N., Neto, C.C.D., Goulart, M.O.F., et al. (2011). Insecticide resistance in Aedes aegypti populations from Ceara, Brazil. Parasite Vector *4*.
101. Linss, J.G., Brito, L.P., Garcia, G.A., Araki, A.S., Bruno, R.V., Lima, J.B., Valle, D., and Martins, A.J. (2014). Distribution and dissemination of the Val1016Ile and Phe1534Cys Kdr mutations in Aedes aegypti Brazilian natural populations. Parasit Vectors *7*, 25.
102. Maestre-Serrano, R., Gomez-Camargo, D., Ponce-Garcia, G., and Flores, A.E. (2014). Susceptibility to insecticides and resistance mechanisms in Aedes aegypti from the Colombian Caribbean Region. Pesticide Biochemistry and Physiology *116*, 63-73.
103. Marcombe, S., Poupardin, R., Darriet, F., Reynaud, S., Bonnet, J., Strode, C., Brengues, C., Yebakima, A., Ranson, H., Corbel, V., et al. (2009). Exploring the molecular basis of insecticide resistance in the dengue vector Aedes aegypti: a case study in Martinique Island (French West Indies). Bmc Genomics *10*.
104. Ponce-Garcia, G., Del Rio-Galvan, S., Barrera, R., Saavedra-Rodriguez, K., Villanueva-Segura, K., Felix, G., Amador, M., and Flores, A.E. (2016). Knockdown Resistance Mutations in Aedes aegypti (Diptera: Culicidae) From Puerto Rico. Journal of Medical Entomology *53*, 1410-1414.
105. Bariami, V., Jones, C.M., Poupardin, R., Vontas, J., and Ranson, H. (2012). Gene Amplification, ABC Transporters and Cytochrome P450s: Unraveling the Molecular Basis of Pyrethroid Resistance in the Dengue Vector, Aedes aegypti. Plos Neglect Trop D *6*.
106. McAllister, J.C., Godsey, M.S., and Scott, M.L. (2012). Pyrethroid resistance in Aedes aegypti and Aedes albopictus from Port-au-Prince, Haiti. Journal of Vector Ecology *37*, 325-332.
107. Francis, S., Saavedra-Rodriguez, K., Perera, R., Paine, M., Black, W.C., and Delgoda, R. (2017). Insecticide resistance to permethrin and malathion and associated mechanisms in Aedes aegypti mosquitoes from St. Andrew Jamaica. Plos One *12*.
108. Yoon, K.S., Kwon, D.H., Strycharz, J.P., Hollingsworth, C.S., Lee, S.H., and Clark, J.M. (2008). Biochemical and Molecular Analysis of Deltamethrin Resistance in the Common Bed Bug (Hemiptera: Cimicidae). Journal of Medical Entomology *45*, 1092-1101.

109. Park, Y., Taylor, M.F.J., and Feyereisen, R. (1997). A valine421 to methionine mutation in IS6 of the hscp voltage-gated sodium channel associated with pyrethroid resistance in Heliothis virescens F. Biochem Bioph Res Co *239*, 688-691.
110. Hopkins, B.W., and Pietrantonio, P.V. (2010). The Helicoverpa zea (Boddie) (Lepidoptera: Noctuidae) voltage-gated sodium channel and mutations associated with pyrethroid resistance in field-collected adult males. Insect Biochem Molec *40*, 385-393.
111. Kuri-Morales, P.A., Correa-Morales, F., Gonzalez-Acosta, C., Moreno-Garcia, M., Santos-Luna, R., Roman-Perez, S., Salazar-Penagos, F., Lombera-Gonzalez, M., Sanchez-Tejeda, G., and Gonzalez-Roldan, J.F. (2018). Insecticide susceptibility status in Mexican populations of Stegomyia aegypti (= Aedes aegypti): a nationwide assessment. Med Vet Entomol *32*, 162-174.
112. Martins, A.J., Ribeiro, C.D., Bellinato, D.F., Peixoto, A.A., Valle, D., and Lima, J.B. (2012). Effect of insecticide resistance on development, longevity and reproduction of field or laboratory selected Aedes aegypti populations. PLoS One *7*, e31889.
113. Grossman, M.K., Uc-Puc, V., Rodriguez, J., Cutler, D.J., Morran, L.T., Manrique-Saide, P., and Vazquez-Prokopec, G.M. (2018). Restoration of pyrethroid susceptibility in a highly resistant Aedes aegypti population. Biol Letters *14*.
114. Chang, C., Huang, X.Y., Chang, P.C., Wu, H.H., and Dai, S.M. (2012). Inheritance and stability of sodium channel mutations associated with permethrin knockdown resistance in Aedes aegypti. Pesticide Biochemistry and Physiology *104*, 136-142.
115. Lozano-Fuentes, S., Saavedra-Rodriguez, K., Black, W.C.t., and Eisen, L. (2012). QCal: a software application for the calculation of dose-response curves in insecticide resistance bioassays. J Am Mosq Control Assoc *28*, 59-61.
116. Saavedra-Rodriguez, K., Suarez, A.F., Salas, I.F., Strode, C., Ranson, H., Hemingway, J., and Black, W.C. (2012). Transcription of detoxification genes after permethrin selection in the mosquito Aedes aegypti. Insect Molecular Biology *21*, 61-77.
117. Strode, C., Wondji, C.S., David, J.P., Hawkes, N.J., Lumjuan, N., Nelson, D.R., Drane, D.R., Karunaratne, S.H.P.P., Hemingway, J., Black, W.C., et al. (2008). Genomic analysis of detoxification genes in the mosquito Aedes aegypti. Insect Biochem Molec *38*, 113-123.
118. Bona, A.C., Chitolina, R.F., Fermino, M.L., de Castro Poncio, L., Weiss, A., Lima, J.B., Paldi, N., Bernardes, E.S., Henen, J., and Maori, E. (2016). Larval application of sodium channel homologous dsRNA restores pyrethroid insecticide susceptibility in a resistant adult mosquito population. Parasit Vectors *9*, 397.
119. Chapadense, F.G., Fernandes, E.K., Lima, J.B., Martins, A.J., Silva, L.C., Rocha, W.T., Santos, A.H., and Cravo, P. (2015). Phenotypic and genotypic profile of pyrethroid resistance in populations of the mosquito Aedes aegypti from Goiania, Central West Brazil. Rev Soc Bras Med Trop *48*, 607-609.

Appendix

Supplemental Information Chapter 2

Table S2.1. Location, collection year, sample size and I1,016 and C1,534 genotypes. VV=V1,016 homozygotes, VI=V1,016/I1,016 heterozygotes, II=I1,016 homozygote, FF=F1,534 homozygotes, FC=F1,534/C1,534 heterozygotes, CC=C1,534 homozygotes for *Ae.aegypti* in Mexico from 1996 to 2012.

State	City (Latitude/Longitude)	Year	Sample size	VV			VI			II		
				FF	FC	CC	FF	FC	CC	FF	FC	CC
Texas (U.S.A.)												
	Houston (29.75944/-95.36193)	1999	47	47	0	0	0	0	0	0	0	0
Tamaulipas												
	Nuevo Laredo (27.5/-96.4667)	2000	50	49	0	0	1	0	0	0	0	0
	Miguel Aleman (26.399543/-99.031043)	1999	47	47	0	0	0	0	0	0	0	0
Nuevo Leon												
	Monterrey (25.6667/-100.30000)	1999	47	47	0	0	0	0	0	0	0	0
		2008	47	3	7	6	0	9	14	0	1	7
Veracruz												
	Panuco (22.05346/-98.18661)	2002	50	26	5	0	0	16	2	0	0	1
	Tuxpan (20.956275/-97.406467)	2012	54	0	0	1	0	0	24	0	0	29
	Tantoyuca (21.34176/-98.22774)	2000	47	47	0	0	0	0	0	0	0	0
		2002	50	50	0	0	0	0	0	0	0	0
		2003	41	40	0	0	1	0	0	0	0	0
		2008	47	3	6	1	0	12	7	0	0	18
	Poza Rica (21.34366/-97.47079)	2000	46	46	0	0	0	0	0	0	0	0
		2002	47	47	0	0	0	0	0	0	0	0
		2003	50	32	16	0	0	1	0	0	0	1
		2008	39	1	2	2	0	15	1	0	0	18
		2012	38	0	0	1	1	1	11	0	0	24
	Martınez de la Torre (20.04999/-97.03883)	2000	47	47	0	0	0	0	0	0	0	0
		2002	47	39	0	0	8	0	0	0	0	0
		2003	30	24	3	0	0	3	0	0	0	0
		2008	48	0	2	7	0	3	21	0	0	15
		2012	54	0	0	0	0	0	15	0	1	38
	Zempoala (19.44489/-92.90000)	2000	47	46	1	0	0	0	0	0	0	0
		2002	47	46	1	0	0	0	0	0	0	0
		2003	30	30	0	0	0	0	0	0	0	0
		2012	54	0	0	4	0	2	17	1	4	26
	Veracruz (19.16508/-96.21625)	2008	46	0	0	7	0	0	22	0	0	17
		2012	54	0	0	0	1	1	18	1	0	33
	Alvarado (18.77422/-95.76356)	2000	47	46	1	0	0	0	0	0	0	0
		2002	50	37	5	0	7	1	0	0	0	0
		2003	49	45	3	1	0	0	0	0	0	0
		2012	54	0	2	6	2	1	30	0	0	13
	Acayucan (17.96196/-94.41255)	2002	47	45	1	0	0	1	0	0	0	0
	Cosoleacaque (17.96196/-94.53605)	2000	47	40	0	0	0	7	0	0	0	0
		2002	47	39	0	0	1	7	0	0	0	0
		2008	47	16	13	4	2	6	3	0	0	3

(Continued)

Table S2.1. (Continued)

State	City (Latitude/Longitude)	Year	Sample size	VV			VI			II		
				FF	FC	CC	FF	FC	CC	FF	FC	CC
	Minatitlan (17.97972/-94.54083)	2002	50	44	2	0	0	4	0	0	0	0
		2003	45	43	2	0	0	0	0	0	0	0
	Coatzacoalcos (18.14081/-94.4131)	2002	50	48	0	0	0	2	0	0	0	0
		2003	47	43	2	2	0	0	0	0	0	0
		2008	50	0	0	23	0	0	27	0	0	0
		2012	54	0	0	9	0	1	27	1	0	16
Tabasco												
	Villahermosa (18/-92.90000)	2000	47	47	0	0	0	0	0	0	0	0
Campeche												
	Ciudad del Carmen (18.641496/-91.82075)	2000	47	47	0	0	0	0	0	0	0	0
	Campeche (19.845446/-90.523673)	2000	47	47	0	0	0	0	0	0	0	0
Yucatan												
	Merida (21.0124/-89.63083)	2000	47	47	0	0	0	0	0	0	0	0
		2007	47	6	2	0	1	27	1	0	4	6
	Merida-Center (20.9519/-89.6408)	2000	47	47	0	0	0	0	0	0	0	0
	Merida-East	2000	47	47	0	0	0	0	0	0	0	0
	Merida-North	2000	47	47	0	0	0	0	0	0	0	0
	Merida-South	2000	37	37	0	0	0	0	0	0	0	0
	Merida-West	2000	47	47	0	0	0	0	0	0	0	0
Quintana Roo												
	Cancun I (21.14/-86.8800)	2000	47	47	0	0	0	0	0	0	0	0
	Cancun II (21.14/-86.8800)	2000	36	36	0	0	0	0	0	0	0	0
	Chetumal-Calderitas (18.5/-88.30000)	2007	47	18	2	1	0	16	2	0	1	7
	Chetumal-Lagunitas (18.50814/-88.29721)	2007	40	1	0	0	1	5	4	0	1	28
	Chetumal-Lazaro Cardenas	2007	47	1	4	1	2	10	14	0	0	15
	Chetumal-Antorchistas	2008	30	11	1	0	0	15	0	0	0	3
	Chetumal-Solidaridad	2008	47	18	3	0	0	22	2	1	0	1
Chiapas												
	Ciudad Hidalgo (14.67902/-92. 15102)	2006	47	32	3	0	0	9	0	0	1	2
		2008	44	2	13	14	3	8	4	0	0	0
	Motozintla (15.37056/-92.24789)	2006	47	46	0	0	1	0	0	0	0	0
		2008	47	24	21	2	0	0	0	0	0	0
	Rio Florido (14.855625/-92.342744)	2006	47	35	1	0	0	4	5	0	0	2
		2008	46	6	16	20	0	1	3	0	0	0
	Puerto Chiapas (14.705707/-92.396214)	2006	48	42	6	0	0	0	0	0	0	0
		2008	47	4	21	22	0	0	0	0	0	0
	Mazatan (14.8615/-92.44862)	2006	47	37	10	0	0	0	0	0	0	0
		2008	47	4	25	9	1	3	5	0	0	0
	Huehuetan (15.01996/-92.39306)	2006	47	47	0	0	0	0	0	0	0	0
		2008	47	5	28	13	0	1	0	0	0	0
	Huixtla (15.14116/-92.46021)	2006	47	42	3	0	2	0	0	0	0	0
		2008	46	4	5	37	0	0	0	0	0	0

(Continued)

Table S2.1. (Continued)

State	City (Latitude/Longitude)	Year	Sample size	VV			VI			II		
				FF	FC	CC	FF	FC	CC	FF	FC	CC
	Escuintla (15.32909/-92.66992)	2006	47	11	12	1	0	16	1	0	0	6
		2008	45	0	6	32	0	1	6	0	0	0
	Mapastepec (15.43309/-92.89723)	2006	47	28	18	1	0	0	0	0	0	0
		2008	47	20	20	7	0	0	0	0	0	0
	Pijijiapan (15.68546/-93.21236)	2006	47	26	17	4	0	0	0	0	0	0
		2008	47	4	30	13	0	0	0	0	0	0
	Tapachula I (14.91368/-92.24116)	2000	47	46	1	0	0	0	0	0	0	0
	Tapachula II	2000	37	36	1	0	0	0	0	0	0	0
Oaxaca												
	Puerto Escondido (15.865535/-97.069447)	2000	47	47	0	0	0	0	0	0	0	0
Guerrero												
	Coyuca de Benitez (17.008464/-100.085473)	2000	47	44	3	0	0	0	0	0	0	0
	Ixtapa (17.660628/-101.601346)	2000	47	47	0	0	0	0	0	0	0	0
Michoacan												
	Lazaro Cardenas (17.959826/-102.191412)	2000	47	47	0	0	0	0	0	0	0	0
Jalisco												
	Puerto Vallarta (20.622018/-	2000	50	50	0	0	0	0	0	0	0	0
Sinaloa												
	Mazatlan (23.2467/-106.43318)	2000	47	47	0	0	0	0	0	0	0	0
Sonora												
	Hermosillo (29.089186/-110.96133)	2000	47	47	0	0	0	0	0	0	0	0
Total	87 collections	4,039										

Table S2.2. Frequencies of I1,016 and C1,534 allele and the 95% Highest Density Intervals around these frequencies. F_{IS} and the associated probabilities from the χ^2 test to test whether F_{IS}=0.

State	City	Year	Ile1,016	95% HDI	FIS	Sig.	Cys1,534	95% HDI	FIS	Sig.
Texas (U.S.A.)										
	Houston	1999	0.000	(0.007–0.031)			0.000	(0.007–0.031)		
Tamaulipas										
	Nuevo Laredo	2000	0.010a	(0.014–0.037)			0.000	(0.007–0.029)		
	Miguel Aleman	1999	0.000	(0.007–0.031)			0.000	(0.007–0.031)		
Nuevo Leon										
	Monterrey	1999	0.000	(0.007–0.031)			0.000	(0.007–0.031)		
		2008	0.410	(0.096–0.100)	-0.008		0.760	(0.093–0.079)	0.021	
Veracruz										
	Panuco	2002	0.200	(0.070–0.085)	-0.125		0.270	(0.080–0.092)	-0.065	
	Tuxpan	2012	0.760	(0.086–0.074)			1.000	(0.027–0.006)		
	Tantoyuca	2000	0.000	(0.007–0.031)			0.000	(0.007–0.031)		
		2002	0.000	(0.007–0.029)			0.000	(0.007–0.029)		
		2003	0.010a	(0.017–0.045)			0.000	(0.008–0.035)		
		2008	0.590	(0.100–0.096)	0.167		0.740	(0.093–0.081)	-0.007	
	Poza Rica	2000	0.000	(0.007–0.031)			0.000	(0.007–0.031)		
		2002	0.000	(0.007–0.031)			0.000	(0.007–0.031)		
		2003	0.030	(0.025–0.048)	0.656	***	0.190	(0.069–0.084)	-0.105	
		2008	0.670	(0.108–0.097)	0.082		0.760	(0.102–0.086)	-0.183	
		2012	0.800	(0.098–0.079)	-0.08		0.960	(0.062–0.033)	0.653	***
	Martınez de la Torre	2000	0.000	(0.007–0.031)			0.000	(0.007–0.031)		
		2002	0.090a	(0.047–0.068)			0.000	(0.007–0.031)		
		2003	0.050	(0.042–0.077)	-0.053		0.100	(0.061–0.093)	-0.111	
		2008	0.560	(0.099–0.096)	-0.016		0.950	(0.058–0.035)	-0.055	
		2012	0.860	(0.073–0.057)	-0.161		0.990	(0.035–0.013)	-0.009	
	Zempoala	2000	0.000	(0.007–0.031)			0.010	(0.015–0.040)	-0.011	
		2002	0.000	(0.007–0.031)			0.010	(0.015–0.040)	-0.011	
		2003	0.000	(0.011–0.047)			0.000	(0.011–0.047)		
		2012	0.750	(0.086–0.075)	0.062		0.930	(0.059–0.041)	0.190	
	Veracruz	2008	0.610	(0.101–0.095)			1.000	(0.032–0.007)		
		2012	0.810	(0.080–0.066)	-0.227		0.950	(0.052–0.031)	0.790	***
	Alvarado	2000	0.000	(0.007–0.031)			0.010	(0.015–0.040)	-0.011	
		2002	0.080a	(0.066–0.080)	-0.087		0.060	(0.037–0.059)	-0.064	
		2003	0.000	(0.007–0.030)			0.050	(0.034–0.057)	0.368	*
		2012	0.550	(0.094–0.091)	-0.233		0.940	(0.058–0.038)	0.542	***
	Acayucan	2002	0.010	(0.015–0.040)	-0.011		0.020	(0.021–0.046)	-0.022	
	Cosoleacaque	2000	0.070	(0.043–0.065)	-0.080		0.070	(0.043–0.065)	-0.080	
		2002	0.090a	(0.047–0.068)	-0.093		0.070	(0.043–0.065)	-0.080	
		2008	0.180	(0.069–0.086)	0.210		0.410	(0.095–0.100)	0.167	
	Minatitlan	2002	0.040	(0.030–0.052)	-0.042		0.060	(0.037–0.059)	-0.064	
		2003	0.000	(0.007–0.032)			0.020	(0.022–0.048)	-0.023	

(Continued)

Table S2.2 (Continued)

State	City	Year	Ile1,016	95% HDI	FIS	Sig.	Cys1,534	95% HDI	FIS	Sig.
	Coatzacoalcos	2002	0.020	(0.020–0.043)	-0.020		0.020	(0.020–0.043)	-0.020	
		2003	0.000	(0.007–0.031)			0.060	(0.040–0.062)	0.644	***
		2008	0.270	(0.080–0.092)	-0.370		1.000	(0.029–0.007)		
		2012	0.570	(0.094–0.090)	-0.060		0.970	(0.045–0.024)	0.657	***
Tabasco										
	Villahermosa	2000	0.000	(0.007–0.031)			0.000	(0.007–0.031)		
Campeche										
	Ciudad del Carmen	2000	0.000	(0.007–0.031)			0.000	(0.007–0.031)		
	Campeche	2000	0.000	(0.007–0.031)			0.000	(0.007–0.031)		
Yucatan										
	Merida	2000	0.000	(0.007–0.031)			0.000	(0.007–0.031)		
		2007	0.52a	(0.100–0.099)	-0.236		0.500	(0.099–0.099)	-0.404	**
	Merida-Center	2000	0.000	(0.007–0.031)			0.000	(0.007–0.031)		
	Merida-East	2000	0.000	(0.007–0.031)			0.000	(0.007–0.031)		
	Merida-North	2000	0.000	(0.007–0.031)			0.000	(0.007–0.031)		
	Merida-South	2000	0.000	(0.009–0.039)			0.000	(0.009–0.039)		
	Merida-West	2000	0.000	(0.007–0.031)			0.000	(0.007–0.031)		
Quintana Roo										
	Cancun I	2000	0.000	(0.007–0.031)			0.000	(0.007–0.031)		
	Cancun II	2000	0.000	(0.009–0.040)			0.000	(0.009–0.040)		
	Chetumal-Calderitas	2007	0.360	(0.092–0.099)	0.170		0.410	(0.096–0.100)	0.167	
	Chetumal-Lagunitas	2007	0.850	(0.089–0.067)	0.020		0.880	(0.084–0.061)	0.314	*
	Chetumal-Cardenas	2007	0.600	(0.100–0.095)	-0.148		0.790	(0.089–0.075)	0.111	
	Chetumal-Antorchistas	2008	0.350	(0.112–0.124)	-0.099		0.370	(0.113–0.124)	-0.148	
	Chetumal-Solidaridad	2008	0.300	(0.086–0.096)	-0.221		0.330	(0.089–0.098)	-0.203	
Chiapas										
	Ciudad Hidalgo	2006	0.160	(0.065–0.083)	0.286	*	0.180	(0.069–0.086)	0.066	
		2008	0.170	(0.069–0.087)	-0.205		0.650	(0.102–0.094)	-0.046	
	Motozintla	2006	0.010	(0.015–0.040)			0.000	(0.007–0.031)		
		2008	0.000	(0.007–0.031)			0.270	(0.082–0.094)	-0.144	
	Rio Florido	2006	0.140	(0.060–0.079)	0.197		0.200	(0.073–0.088)	0.670	***
		2008	0.040	(0.032–0.056)	-0.045		0.680	(0.098–0.089)	0.144	
	Puerto Chiapas	2006	0.000	(0.007–0.030)			0.060	(0.039–0.061)	-0.067	
		2008	0.000	(0.007–0.031)			0.690	(0.097–0.087)	-0.047	
	Mazatan	2006	0.000	(0.007–0.031)			0.110	(0.052–0.073)	-0.119	
		2008	0.100	(0.050–0.071)	-0.106		0.600	(0.100–0.095)	-0.237	
	Huehuetan	2006	0.000	(0.007–0.031)			0.000	(0.007–0.031)		
		2008	0.010	(0.015–0.040)	-0.011		0.590	(0.100–0.096)	-0.271	
	Huixtla	2006	0.020	(0.021–0.046)	-0.022		0.030	(0.027–0.051)	-0.033	
		2008	0.000	(0.007–0.031)			0.860	(0.081–0.062)	0.552	
	Escuintla	2006	0.310	(0.087–0.097)	0.152		0.470	(0.098–0.100)	-0.196	
		2008	0.080	(0.045–0.068)	-0.084		0.920	(0.068–0.045)	-0.084	
	Mapastepec	2006	0.000	(0.007–0.031)			0.210	(0.074–0.089)	-0.143	
		2008	0.000	(0.007–0.031)			0.360	(0.092–0.099)	0.078	
	Pijijiapan	2006	0.000	(0.007–0.031)			0.270	(0.082–0.094)	0.074	
		2008	0.000	(0.007–0.031)			0.600	(0.100–0.095)	-0.325	*
	Tapachula I	2000	0.000	(0.007–0.031)			0.010	(0.015–0.040)	-0.011	
	Tapachula II	2000	0.000	(0.009–0.039)			0.010	(0.019–0.050)	-0.014	

(Continued)

Table S2.2 (Continued)

State	City	Year	Ile1,016	95% HDI	FIS	Sig.	Cys1,534	95% HDI	FIS	Sig.
Oaxaca										
	Puerto Escondido	2000	0.000	(0.007–0.031)			0.000	(0.007–0.031)		
Guerrero										
	Coyuca de Benitez	2000	0.000	(0.007–0.031)			0.030	(0.027–0.051)	-0.033	
	Ixtapa	2000	0.000	(0.007–0.031)			0.000	(0.007–0.031)		
Michoacan										
	Lazaro Cardenas	2000	0.000	(0.007–0.031)			0.000	(0.007–0.031)		
Jalisco										
	Puerto Vallarta	2000	0.000	(0.007–0.029)			0.000	(0.007–0.029)		
Sinaloa										
	Mazatlan	2000	0.000	(0.007–0.031)			0.000	(0.007–0.031)		
Sonora										
	Hermosillo	2000	0.000	(0.007–0.031)			0.000	(0.007–0.031)		

Table S2.3. Linkage disequilibrium between I1016 and C1,534 mutations in *Aedes aegypti* in Mexican populations.

State	City	Year	Rij	χ2	Prob
Nuevo Leon					
	Monterrey	2008	0.412	8.09	0.004
Veracruz					
	Panuco	2002	0.839	28.76	0
	Tantoyuca	2008	0.756	31.13	0
	Poza Rica	2003	0.483	17.32	0
		2008	0.716	17.61	0
		2012	0.257	3.81	0.051
	Martınez de la Torre	2003	0.69	12.02	0.001
		2008	0.263	3.08	0.079
		2012	0.087	0.34	0.56
	Zempoala	2012	0.148	1.49	0.222
	Veracruz	2012	0.113	0.95	0.33
	Alvarado	2002	0.007	0	1
		2012	0.187	2.24	0.134
	Acayucan	2002	0.715	23.23	0
	Cosoleacaque	2000	1	41.49	0
		2002	0.944	34.91	0
		2008	0.477	15.11	0
	Minatitlan	2002	0.815	29.78	0
	Coatzacoalcos	2002	1	49.96	0
		2012	0.145	1.76	0.185
Yucatan					
	Merida	2007	0.774	12.82	0
	Quintana Roo				
	Chetumal-Calderitas	2007	0.856	47.03	0
	Chetumal-Lagunitas	2007	0.75	30.17	0
	Chetumal-Lazaro Cardenas	2007	0.571	16.4	0
	Chetumal-Antorchistas	2008	0.993	22.71	0
	Chetumal-Solidaridad	2008	0.74	15.97	0
Chiapas					
	Ciudad Hidalgo	2006	0.894	51.5	0
	Rio Florido	2006	0.929	81.01	0
		2008	0.171	1.46	0.227
	Mazatan	2008	0.207	1.38	0.24
	Huehuetan	2008	0.043	0.06	0.806
	Huixtla	2006	0.056	0.14	0.708
	Escuintla	2006	0.728	23.06	0
		2008	0.015	0.01	0.92

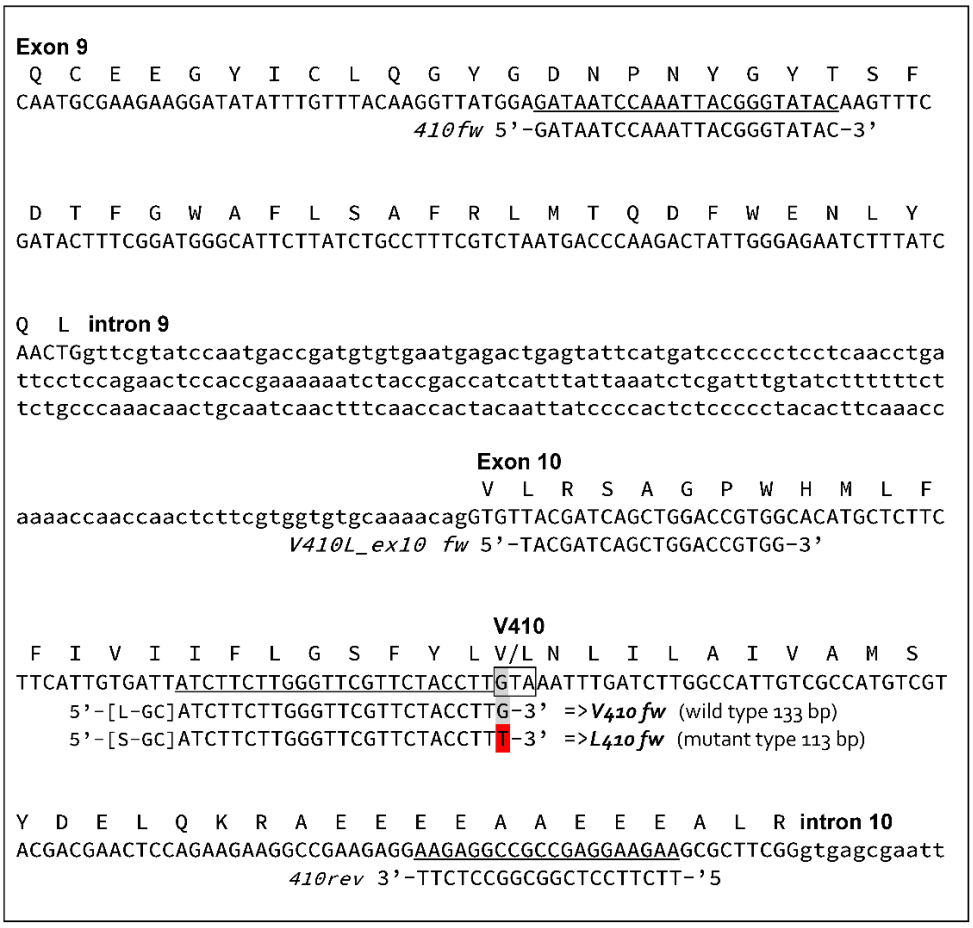


Figure S3.1. Genomic and amino acid sequences of *vgsc* exons 9 and 10. The *410fw* and *410rev* primers were used to amplify a 500 bp region flanking exon 9 and 10. *V410L_ex10 fw* and *410rev* pair was used for Sanger sequencing. The V410L substitution is highlighted in red and the codon is bordered. Allele-specific melting curve primers (*V410fw* and *L410fw*) and reverse primers (*410rev*) are shown below the underlined genomic sequence. L- GC corresponds to a 26 mer GC rich-tail and S-GC to a 6 mer GC rich-tail attached to the 5'-primer sequence.

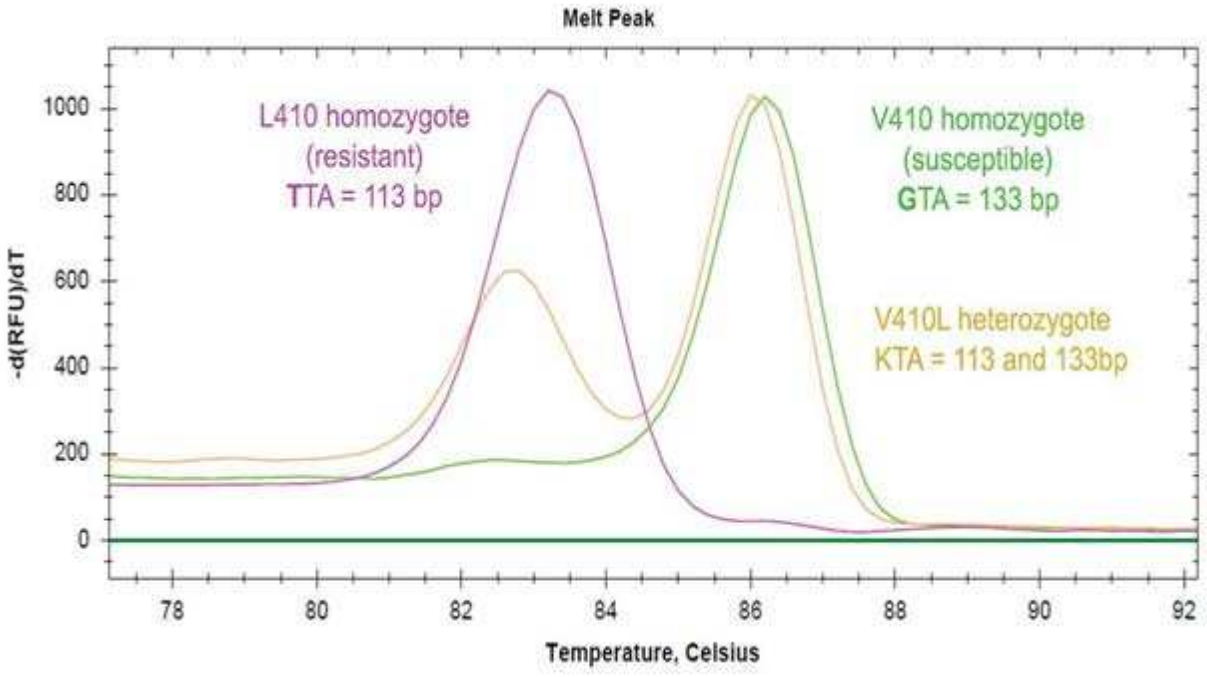


Figure S3.2. Melting curve peaks obtained for V410L genotypes. Representative patterns for the LL_{410} resistant homozygote (a single peak at 82.5ºC), VV_{410} susceptible homozygote (a single peak at 87ºC) and a VL_{410} heterozygote (at 82.5ºC and 87ºC) mosquito. Amplicon sizes are shown for each allele.

Table S3.1. ***Aedes aegypti*** **collections from Mexico.** The region, site, year and number of mosquitoes genotyped. Geographical coordinates are published in Garcia et al., 2009 and Vera Maloof et al., 2015.

Region	Site (City and State)	Year	n
Eastern			
	Poza Rica, Veracruz	2000	46
		2003	47
		2008	39
		2012	37
	Martinez de la Torre,Veracruz	2000	46
		2002	42
		2003	30
		2008	48
		2012	44
	Zempoala, Veraruz	2000	47
		2002	47
		2003	30
		2012	52
	Coatzacoalcos, Veracruz	2002	50
		2003	48
		2008	48
		2012	45
Southeastern			
	Merida, Yucatan	2000	48
		2005	42
		2007	47
		2013	96
Southwestern			
	Tapachula, Chiapas	2000	47
		2006	48
		2014	47
		2016	50

Table S3.2. Phenotypic repose of *Ae. aegypti* exposed to an LC_{50} of permethrin (pyrethroid type 1) or deltamethrin (pyrethroid type 2) in a bottle bioassay. After an insecticide exposure of 1 h, active mosquitoes were separated from inactive mosquitoes and transferred to different containers. Following 4 h, three possible phenotypes were scored: 'Alive' were mosquitoes active at 1 h of exposure and still active at 4 h; 'recovered mosquitoes were initially in the inactive group but recovered activity within 4 h and 'dead' mosquitoes were inactive at 1 h and continued to be inactive at 4 h port-exposure.

	Number of individuals			
Insecticide	**Alive**	**Recovered**	**Dead**	**Total**
Permethrin 25 µg	108 (18%)	96 (16%)	411 (67%)	615
Deltamethrin 3 µg	149 (38%)	93 (24%)	148 (38%)	390

Table S.3.3 Frequency of tri-locus genotypes at loci 410/1,016/1,534 in Mexico at four periods of time. Observed and expected frequencies are shown for each genotype; *indicate the genotypes were not observed. Bold numbers shown the genotypes that exceeded expected.

No. of resistant alleles	Tri-locus genotype	2000		2002-2005		2006-2008		2012-2016	
		obs.	exp.	obs.	exp.	obs.	exp.	obs.	exp.
0	VV/VV/FF	231	231	299	289	9	0.8	0	0
1	VV/VV/FC	2	1.99	28	33.6	13	6.69	4	0.16
1	VV/VI/FF	0	0	8	11.9	0	1.28	1	0.01
1	VL/VV/FF	0	0	2	7.6	1	1.26	0	0.01
2	VV/VV/CC	0	0	2	0.98	**45**	**14**	**31**	**2.5**
2	VV/VI/FC	0	0	1	1.39	3	10.68	0	0.79
2	VV/II/FF	0	0	0	0.12	0	0.51	1	0.02
2	VL/VV/FC	0	0	0	0.89	2	10.59	0	0.76
2	VL/VI/FF	0	0	0	0.31	1	2.02	0	0.06
2	LL/VV/FF	0	0	1	0.05	0	0.5	0	0.01
3	VL/VI/FC	0	0	5	0.04	**42**	**16.9**	7	3.79
3	VV/VI/CC*	0	0	0	0.04	0	22.36	0	12.53
3	VV/II/FC*	0	0	0	0.01	0	4.26	0	0.99
3	VL/VV/CC*	0	0	0	0.03	0	22.16	0	11.97
3	VL/II/FF*	0	0	0	0	0	0.81	0	0.08
3	LL/VV/FC*	0	0	0	0.01	0	4.19	0	0.91
3	LL/VI/FF*	0	0	0	0	0	0.8	0	0.07
4	VV/II/CC	0	0	0	0	0	8.93	2	15.63
4	VL/VI/CC	0	0	0	0	**57**	**35.4**	**128**	**59.8**
4	VL/II/FC	0	0	0	0	1	6.75	0	4.73
4	LL/VV/CC	0	0	0	0	1	8.76	0	14.28
4	LL/VI/FC	0	0	0	0	3	6.69	1	4.52
4	LL/II/FF*	0	0	0	0	0	0.32	0	0.09
5	VL/II/CC	0	0	0	0	5	14.12	8	74.55
5	LL/VI/CC	0	0	0	0	0	14	7	71.24
5	LL/II/FC	0	0	0	0	0	2.67	7	5.64
6	LL/II/CC	0	0	0	0	**40**	**5.6**	**177**	**88.9**
	Total	233	233	346	346	223	223	374	374

Supplemental Information Chapter 4

Table 4.1S. Collection site, generation, sample size and counts of V1,016I and F1,534C di-locus genotypes.

Site	Generation	Rep	N	1,534/1,016 di-locus genotype								
				VV/FF	VV/FC	VV/CC	IV/FF	IV/FC	IV/CC	II/FF	II/FC	II/CC
Acp												
	F1	1	50	0	0	0	0	0	12	1	0	37
		2	50	0	0	0	0	0	16	0	0	34
		3	51	0	0	3	0	0	16	0	0	32
		Total	**151**	**0**	**0**	**3**	**0**	**0**	**44**	**1**	**0**	**103**
	F2	1	50	1	0	4	0	0	13	0	0	32
		2	50	3	0	7	0	0	11	0	0	29
		3	50	1	0	1	0	0	19	0	0	29
		Total	**150**	**5**	**0**	**12**	**0**	**0**	**43**	**0**	**0**	**90**
	F3	1	50	0	0	2	0	0	18	0	0	30
		2	50	1	0	2	0	2	21	0	0	24
		3	50	2	0	1	0	8	19	0	0	20
		Total	**150**	**3**	**0**	**5**	**0**	**10**	**58**	**0**	**0**	**74**
	F4	1	50	0	2	4	0	2	20	0	0	22
		2	50	0	2	0	0	7	18	0	1	22
		3	50	0	3	3	0	4	18	0	0	22
		Total	**150**	**0**	**7**	**7**	**0**	**13**	**56**	**0**	**1**	**66**
	F5	1	50	0	3	3	0	5	15	0	0	24
		2	50	0	3	1	0	4	20	0	0	22
		3	50	0	4	0	0	4	17	0	0	25
		Total	**150**	**0**	**10**	**4**	**0**	**13**	**52**	**0**	**0**	**71**
	F6	1	50	0	8	0	0	12	9	0	1	20
		2	50	0	3	1	0	10	13	0	0	23
		3	50	0	3	5	0	8	21	0	0	13
		Total	**150**	**0**	**14**	**6**	**0**	**30**	**43**	**0**	**1**	**56**
	F7	1	50	11	0	0	0	29	0	0	0	10
		2	50	2	1	9	0	11	18	0	0	9
		3	50	12	1	1	0	22	3	0	0	11
		Total	**150**	**25**	**2**	**10**	**0**	**62**	**21**	**0**	**0**	**30**
	F8	1	50	22	3	0	0	13	5	0	0	7
		2	50	14	7	0	0	20	6	0	0	3
		3	50	21	5	0	0	20	0	0	0	4
		Total	**150**	**57**	**15**	**0**	**0**	**53**	**11**	**0**	**0**	**14**
Tap												
	F1	1	50	0	0	7	0	0	25	0	0	18
		2	50	0	0	9	0	0	26	0	0	15
		3	50	0	3	3	1	1	21	0	0	21
		Total	**150**	**0**	**3**	**19**	**1**	**1**	**72**	**0**	**0**	**54**
	F2	1	50	0	0	2	0	0	19	0	0	29
		2	50	0	0	4	0	0	21	0	0	25
		3	50	0	0	1	0	0	31	0	0	18
		Total	**150**	**0**	**0**	**7**	**0**	**0**	**71**	**0**	**0**	**72**
	F3	1	50	0	0	8	0	0	24	0	0	18
		2	50	0	0	8	0	0	20	0	0	22
		3	50	0	0	8	0	0	23	0	0	19
		Total	**150**	**0**	**0**	**24**	**0**	**0**	**67**	**0**	**0**	**59**
	F4	1	50	0	0	7	0	0	24	0	0	19
		2	50	0	0	5	0	0	30	0	0	15
		3	50	0	0	6	0	0	24	0	0	20
		Total	**150**	**0**	**0**	**18**	**0**	**0**	**78**	**0**	**0**	**54**

Table 4.1S. (Continued)

Site	Generation	Rep	N	1,534/1,016 di-locus genotype								
				VV/FF	VV/FC	VV/CC	IV/FF	IV/FC	IV/CC	II/FF	II/FC	II/CC
	F5	1	50	0	0	12	0	1	30	0	0	7
		2	50	0	0	4	0	0	30	0	2	14
		3	50	0	0	9	0	1	34	0	0	6
		Total	**150**	**0**	**0**	**25**	**0**	**2**	**94**	**0**	**2**	**27**
	F6	1	50	0	0	9	0	0	29	0	0	12
		2	50	0	0	15	0	0	22	0	0	13
		3	50	0	0	7	0	0	27	0	0	16
		Total	**150**	**0**	**0**	**31**	**0**	**0**	**78**	**0**	**0**	**41**
	F7	1	50	0	0	13	0	0	22	0	0	15
		2	50	0	0	7	0	0	24	0	0	19
		3	50	0	0	13	0	0	19	0	0	18
		Total	**150**	**0**	**0**	**33**	**0**	**0**	**65**	**0**	**0**	**52**
	F8	1	50	0	0	13	0	0	15	0	0	22
		2	50	0	1	9	0	0	23	0	0	17
		3	50	0	0	8	0	0	24	0	0	18
		Total	**150**	**0**	**1**	**30**	**0**	**0**	**62**	**0**	**0**	**57**
Mer1												
	F1	1	50	0	0	8	1	10	14	0	0	17
		2	50	0	0	6	0	6	24	0	0	14
		3	18	0	0	2	0	3	6	0	0	7
		Total	**118**	**0**	**0**	**16**	**1**	**19**	**44**	**0**	**0**	**38**
	F2	1	50	0	7	13	0	1	16	0	0	13
		2	50	0	6	9	0	6	20	0	0	9
		3	50	0	3	12	0	4	18	0	0	13
		Total	**150**	**0**	**16**	**34**	**0**	**11**	**54**	**0**	**0**	**35**
	F3	1	50	0	13	6	0	9	6	0	1	15
		2	50	1	0	16	0	1	22	1	0	9
		3	50	0	2	14	0	3	20	0	0	11
		Total	**150**	**1**	**15**	**36**	**0**	**13**	**48**	**1**	**1**	**35**
	F4	1	50	0	5	21	0	1	17	0	0	6
		2	50	0	5	12	0	1	18	0	0	14
		3	50	0	5	14	0	1	23	0	0	7
		Total	**150**	**0**	**15**	**47**	**0**	**3**	**58**	**0**	**0**	**27**
	F5	1	50	0	0	13	0	0	29	0	0	8
		2	50	0	0	16	0	0	25	0	0	9
		3	50	0	0	10	0	3	25	0	0	12
		Total	**150**	**0**	**0**	**39**	**0**	**3**	**79**	**0**	**0**	**29**
	F6	1	50	0	1	10	0	0	29	0	0	10
		2	50	0	1	14	0	2	22	0	1	10
		3	50	0	1	14	0	1	16	0	0	18
		Total	**150**	**0**	**3**	**38**	**0**	**3**	**67**	**0**	**1**	**38**
	F7	1	50	1	1	10	0	2	28	0	0	8
		2	50	3	0	19	0	0	24	0	0	4
		3	50	0	1	18	0	0	21	0	0	10
		Total	**150**	**4**	**2**	**47**	**0**	**2**	**73**	**0**	**0**	**22**
	F8	1	50	1	3	27	0	1	14	0	0	4
		2	50	0	2	13	0	0	25	0	0	10
		3	50	0	3	15	0	0	29	0	0	3
		Total	**150**	**1**	**8**	**55**	**0**	**1**	**68**	**0**	**0**	**17**
Mer2												
	F1	1	50	2	4	7	1	6	13	0	0	17
		2	50	2	2	7	0	9	11	0	0	19
		3	50	1	3	3	0	4	18	0	0	21

Continued

Table 4.1S. (Continued)

Site	Generation	Rep	N	1,534/1,016 di-locus genotype								
				VV/FF	VV/FC	VV/CC	IV/FF	IV/FC	IV/CC	II/FF	II/FC	II/CC
		Total	**150**	**5**	**9**	**17**	**1**	**19**	**42**	**0**	**0**	**57**
	F2	1	50	2	11	7	0	4	10	0	0	16
		2	50	2	8	4	0	8	19	0	0	9
		3	50	4	9	6	4	5	18	0	0	4
		Total	**150**	**8**	**28**	**17**	**4**	**17**	**47**	**0**	**0**	**29**
	F3	1	50	1	10	7	1	8	12	0	0	11
		2	50	1	12	4	0	18	12	0	0	3
		3	50	4	7	8	0	12	8	0	0	11
		Total	**150**	**6**	**29**	**19**	**1**	**38**	**32**	**0**	**0**	**25**
	F4	1	50	2	9	6	0	8	20	0	0	5
		2	50	2	12	8	0	6	10	0	1	11
		3	50	1	9	14	0	6	13	0	4	3
		Total	**150**	**5**	**30**	**28**	**0**	**20**	**43**	**0**	**5**	**19**
	F5	1	50	8	11	0	0	15	9	0	0	7
		2	50	11	10	0	0	12	9	0	0	8
		3	50	15	3	1	1	19	8	0	0	3
		Total	**150**	**34**	**24**	**1**	**1**	**46**	**26**	**0**	**0**	**18**
	F6	1	50	22	9	0	1	12	4	0	0	2
		2	50	13	8	0	0	14	7	0	0	8
		3	50	12	12	2	0	15	3	1	0	5
		Total	**150**	**47**	**29**	**2**	**1**	**41**	**14**	**1**	**0**	**15**
	F7	1	50	12	9	0	0	22	5	0	0	2
		2	50	14	6	5	0	17	5	0	0	3
		3	50	7	12	2	0	18	5	0	0	6
		Total	**150**	**33**	**27**	**7**	**0**	**57**	**15**	**0**	**0**	**11**
	F8	1	50	11	15	2	0	11	8	0	0	3
		2	50	14	4	0	0	19	8	0	0	5
		3	44	9	6	2	1	16	8	0	0	2
		Total	**144**	**34**	**25**	**4**	**1**	**46**	**24**	**0**	**0**	**10**
Mer3												
	F1	1	50	0	0	7	0	0	23	0	0	20
		2	50	0	0	2	0	0	15	0	0	33
		3	50	0	0	1	0	0	23	0	0	26
		Total	**150**	**0**	**0**	**10**	**0**	**0**	**61**	**0**	**0**	**79**
	F2	1	50	0	0	2	0	0	15	0	0	33
		2	50	1	0	5	0	0	16	0	0	28
		3	50	0	0	3	0	0	16	0	0	31
		Total	**150**	**1**	**0**	**10**	**0**	**0**	**47**	**0**	**0**	**92**
	F3	1	50	5	0	5	0	0	17	0	0	23
		2	50	1	0	6	0	0	12	0	0	31
		3	50	0	0	2	0	0	16	0	0	32
		Total	**150**	**6**	**0**	**13**	**0**	**0**	**45**	**0**	**0**	**86**
	F4	1	50	0	0	2	0	0	13	0	0	35
		2	50	0	1	9	0	0	17	0	0	23
		3	50	0	1	7	0	8	9	1	7	17
		Total	**150**	**0**	**2**	**18**	**0**	**8**	**39**	**1**	**7**	**75**
	F5	1	50	0	1	17	0	0	23	0	0	9
		2	50	0	0	11	0	0	26	0	0	13
		3	50	0	0	18	0	0	24	0	0	8
		Total	**150**	**0**	**1**	**46**	**0**	**0**	**73**	**0**	**0**	**30**
	F6	1	50	0	0	17	0	0	22	0	0	11
		2	50	0	0	16	0	0	20	0	0	14
		3	50	0	0	14	0	0	26	0	0	10

Continued

Table 4.1S. (Continued)

Site	Generation	Rep	N	1,534/1,016 di-locus genotype								
				VV/FF	VV/FC	VV/CC	IV/FF	IV/FC	IV/CC	II/FF	II/FC	II/CC
		Total	**150**	**0**	**0**	**47**	**0**	**0**	**68**	**0**	**0**	**35**
	F7	1	50	0	0	17	0	0	28	0	0	5
		2	50	0	0	15	0	1	22	0	0	12
		3	50	0	0	8	0	0	19	0	0	23
		Total	**150**	**0**	**0**	**40**	**0**	**1**	**69**	**0**	**0**	**40**
	F8	1	50	0	0	4	0	0	29	0	0	17
		2	50	0	0	10	0	1	16	0	0	23
		3	50	0	0	4	0	0	28	0	0	18
		Total	**150**	**0**	**0**	**18**	**0**	**1**	**73**	**0**	**0**	**58**
Dz												
	F1	1	50	4	14	3	1	5	15	0	0	8
		2	50	7	12	7	0	10	9	1	0	4
		3	50	3	9	4	0	16	13	0	0	5
		Total	**150**	**14**	**35**	**14**	**1**	**31**	**37**	**1**	**0**	**17**
	F2	1	50	1	0	2	0	0	24	0	0	23
		2	50	1	1	4	0	11	19	0	0	14
		3	50	3	8	6	1	13	15	0	0	4
		Total	**150**	**5**	**9**	**12**	**1**	**24**	**58**	**0**	**0**	**41**
	F3	1	50	5	5	3	0	16	9	1	0	11
		2	50	6	9	2	0	17	12	0	0	4
		3	50	1	11	4	0	15	13	0	0	6
		Total	**150**	**12**	**25**	**9**	**0**	**48**	**34**	**1**	**0**	**21**
	F4	1	50	8	5	0	7	7	20	3	0	0
		2	50	5	5	3	0	14	19	0	1	3
		3	50	1	2	2	3	9	20	0	4	9
		Total	**150**	**14**	**12**	**5**	**10**	**30**	**59**	**3**	**5**	**12**
	F5	1	50	0	10	3	0	11	21	0	0	5
		2	50	0	7	4	0	12	22	0	2	3
		3	50	0	9	5	0	17	9	0	0	10
		Total	**150**	**0**	**26**	**12**	**0**	**40**	**52**	**0**	**2**	**18**
	F6	1	50	3	2	6	0	18	13	0	0	8
		2	50	6	5	2	0	15	11	0	0	11
		3	50	4	9	2	1	12	12	0	0	10
		Total	**150**	**13**	**16**	**10**	**1**	**45**	**36**	**0**	**0**	**29**
	F7	1	50	5	4	8	0	18	12	0	0	3
		2	50	6	11	1	0	17	10	0	0	5
		3	50	4	8	3	1	17	10	0	0	7
		Total	**150**	**15**	**23**	**12**	**1**	**52**	**32**	**0**	**0**	**15**
	F8	1	50	16	7	0	0	19	5	0	0	3
		2	50	9	10	4	0	14	11	0	0	2
		3	50	5	4	9	1	9	15	0	1	6
		Total	**150**	**30**	**21**	**13**	**1**	**42**	**31**	**0**	**1**	**11**
Co												
	F1	1	50	2	2	0	1	4	18	0	0	23
		2	50	10	2	1	5	11	10	0	0	11
		3	50	2	3	2	1	9	11	1	0	21
		Total	**150**	**14**	**7**	**3**	**7**	**24**	**39**	**1**	**0**	**55**
	F2	1	50	0	1	1	1	1	10	0	4	32
		2	50	2	3	3	0	17	10	0	0	15
		3	50	2	3	6	0	23	9	0	0	7
		Total	**150**	**4**	**7**	**10**	**1**	**41**	**29**	**0**	**4**	**54**
	F3	1	50	7	5	1	0	15	8	0	3	11
		2	50	4	7	1	0	16	16	0	1	5

Continued

Table 4.1S. (Continued)

Site	Generation	Rep	N	1,534/1,016 di-locus genotype								
				VV/FF	VV/FC	VV/CC	IV/FF	IV/FC	IV/CC	II/FF	II/FC	II/CC
		3	50	7	4	4	0	13	9	0	0	13
		Total	**150**	**18**	**16**	**6**	**0**	**44**	**33**	**0**	**4**	**29**
	F4	1	50	1	10	0	0	16	16	0	0	7
		2	50	2	7	0	0	24	13	0	0	4
		3	50	10	4	1	0	17	6	0	0	12
		Total	**150**	**13**	**21**	**1**	**0**	**57**	**35**	**0**	**0**	**23**
	F5	1	50	22	13	0	1	11	0	1	0	2
		2	50	24	12	1	0	10	0	0	0	3
		3	50	17	13	0	0	15	0	0	0	5
		Total	**150**	**63**	**38**	**1**	**1**	**36**	**0**	**1**	**0**	**10**
	F6	1	50	29	11	4	0	4	2	0	0	0
		2	50	23	18	2	1	6	0	0	0	0
		3	50	28	11	2	1	5	3	0	0	0
		Total	**150**	**80**	**40**	**8**	**2**	**15**	**5**	**0**	**0**	**0**
	F7	1	50	16	16	1	0	11	5	0	0	1
		2	50	34	7	0	0	7	1	0	0	1
		3	50	38	4	0	0	8	0	0	0	0
		Total	**150**	**88**	**27**	**1**	**0**	**26**	**6**	**0**	**0**	**2**
	F8	1	50	4	3	0	21	3	2	12	4	1
		2	50	18	3	0	21	6	0	2	0	0
		3	50	9	7	4	0	13	10	0	0	7
		Total	**150**	**31**	**13**	**4**	**42**	**22**	**12**	**14**	**4**	**8**
Ac												
	F1	1	50	0	2	2	0	4	9	0	0	33
		2	50	1	1	1	0	5	11	0	0	31
		3	50	2	0	2	0	6	12	0	1	27
		Total	**150**	**3**	**3**	**5**	**0**	**15**	**32**	**0**	**1**	**91**
	F2	1	50	5	3	0	2	17	11	0	0	12
		2	50	0	1	0	1	7	16	0	0	25
		3	50	0	1	1	0	5	11	1	1	30
		Total	**150**	**5**	**5**	**1**	**3**	**29**	**38**	**1**	**1**	**67**
	F3	1	50	5	3	2	1	14	10	0	0	15
		2	50	0	3	0	0	12	8	0	0	27
		3	50	1	1	0	0	7	15	0	0	26
		Total	**150**	**6**	**7**	**2**	**1**	**33**	**33**	**0**	**0**	**68**
	F4	1	50	0	3	2	0	16	14	0	0	15
		2	50	0	2	2	0	12	16	0	0	18
		3	50	3	2	2	0	9	21	0	0	13
		Total	**150**	**3**	**7**	**6**	**0**	**37**	**51**	**0**	**0**	**46**
	F5	1	50	0	2	5	0	5	23	0	0	15
		2	50	0	3	3	0	3	27	0	0	14
		3	50	0	2	6	0	3	27	0	0	12
		Total	**150**	**0**	**7**	**14**	**0**	**11**	**77**	**0**	**0**	**41**
	F6	1	50	1	2	7	0	9	16	0	0	15
		2	50	0	2	3	0	10	21	0	0	14
		3	50	0	3	7	0	7	12	0	0	21
		Total	**150**	**1**	**7**	**17**	**0**	**26**	**49**	**0**	**0**	**50**
	F7	1	50	1	12	3	0	7	19	0	0	8
		2	50	1	9	3	0	11	14	0	0	12
		3	50	1	11	2	0	10	12	0	0	14
		Total	**150**	**3**	**32**	**8**	**0**	**28**	**45**	**0**	**0**	**34**
	F8	1	50	2	13	2	0	6	16	0	0	11
		2	50	1	8	5	0	10	21	0	0	5

Continued

Table 4.1S. (Continued)

Site	Generation	Rep	N	1,534/1,016 di-locus genotype								
				VV/FF	VV/FC	VV/CC	IV/FF	IV/FC	IV/CC	II/FF	II/FC	II/CC
		3	50	1	10	3	0	11	20	1	0	4
		Total	**150**	**4**	**31**	**10**	**0**	**27**	**57**	**1**	**0**	**20**

VV = V1,016 homozygote, VI = V1,016I heterozygote, II = I1,016 homozygote, FF = F1,534 homozygote, FC = F1,534C heterozygote, CC = C1,534 homozygote for *Ae. aegypti* in eight generations of insecticide relaxation.

Table 4.2S. I1,016 and C1,534 allele frequencies of *Ae. aegypti* in eight generations of insecticide relaxation.

Site	G	Rep	I1,016	*P*	95% HDI	F_{IS}	Sig	C1,534	*P*	95% HDI	F_{IS}	Sig
Acp												
	F1	1	0.88		(0.7615-0.9428)	-0.14		0.98		(0.8953-0.9952)	1.00	***
		2	0.84		(0.7143-0.9157)	-0.19		1.00		(0.9302-0.9995)	-	-
		3	0.78		(0.6532-0.8744)	0.07		1.00		(0.9316-0.9995)	-	-
		Total	**0.83**	**0.430**	**(0.7883-0.872)**	**-0.06**		**0.99**	**0.362**	**(0.9764-0.998)**	**1.00**	***
	F2	1	0.77		(0.647-0.8718)	0.27		0.98		(0.8953-0.9952)	1.00	***
		2	0.69		(0.5616-0.8088)	0.49	***	0.94		(0.8379-0.9782)	1.00	***
		3	0.77		(0.647-0.8718)	-0.07		0.98		(0.8953-0.9952)	1.00	***
		Total	**0.74**	**0.563**	**(0.691-0.7893)**	**0.25**	**	**0.97**	**0.437**	**(0.9398-0.9816)**	**1.00**	***
	F3	1	0.78		(0.6532-0.8744)	-0.05		1.00		(0.9302-0.9995)	-	-
		2	0.71		(0.5826-0.825)	-0.12		0.96		(0.8655-0.9877)	0.48	
		3	0.67		(0.541-0.7924)	-0.22		0.88		(0.7615-0.9428)	0.24	
		Total	**0.72**	**0.529**	**(0.6667-0.7677)**	**-0.12**		**0.95**	**0.025**	**(0.9152-0.9667)**	**0.34**	
	F4	1	0.66		(0.5206-0.7756)	0.02		0.96		(0.8655-0.9877)	-0.04	
		2	0.71		(0.5826-0.825)	-0.21		0.9		(0.7859-0.9554)	-0.11	
		3	0.66		(0.5206-0.7756)	0.02		0.93		(0.8379-0.9782)	-0.08	
		Total	**0.677**	**0.759**	**(0.6217-0.727)**	**-0.05**		**0.93**	**0.472**	**(0.8953-0.9536)**	**-0.08**	
	F5	1	0.68		(0.541-0.7924)	0.08		0.92		(0.8115-0.9673)	-0.09	
		2	0.68		(0.541-0.7924)	-0.10		0.93		(0.8379-0.9782)	-0.08	
		3	0.71		(0.5826-0.825)	-0.02		0.92		(0.8115-0.9673)	-0.09	
		Total	**0.69**	**0.882**	**(0.6355-0.7396)**	**-0.01**		**0.92**	**0.907**	**(0.8876-0.9483)**	**-0.08**	
	F6	1	0.63		(0.5006-0.7586)	0.10		0.79		(0.669-0.8869)	-0.27	
		2	0.69		(0.5616-0.8088)	-0.08		0.87		(0.7615-0.9428)	-0.15	
		3	0.55		(0.4222-0.6885)	-0.17		0.89		(0.7859-0.9554)	-0.12	
		Total	**0.62**	**0.346**	**(0.5672-0.6762)**	**-0.04**		**0.85**	**0.313**	**(0.8051-0.8859)**	**-0.18**	
	F7	1	0.49		(0.3658-0.634)	-0.16		0.49		(0.3658-0.634)	-0.16	
		2	0.47		(0.3476-0.6156)	-0.16		0.84		(0.7143-0.9157)	0.11	
		3	0.47		(0.3476-0.6156)	0.00		0.53		(0.4032-0.6706)	0.08	
		Total	**0.48**	**0.974**	**(0.4207-0.5332)**	**-0.11**		**0.62**	**0.001**	**(0.5638-0.673)**	**0.10**	
	F8	1	0.32		(0.2077-0.4589)	0.17		0.4		(0.276-0.5391)	0.33	
		2	0.32		(0.2077-0.4589)	-0.20		0.45		(0.3295-0.5966)	-0.09	
		3	0.28		(0.175-0.4174)	0.01		0.33		(0.2245-0.4793)	-0.13	
		Total	**0.31**	**0.882**	**(0.2572-0.361)**	**0.00**		**0.39**	**0.472**	**(0.3398-0.4497)**	**0.05**	
Tap												
	F1	1	0.61		(0.4806-0.7415)	-0.05		1		(0.9302-0.9995)	-	-
		2	0.56		(0.4222-0.6885)	-0.06		1		(0.9302-0.9995)	-	-
		3	0.65		(0.5206-0.7756)	-0.01		0.94		(0.8379-0.9782)	0.29	
		Total	**0.61**	**0.586**	**(0.5503-0.6602)**	**-0.03**		**0.98**	**0.047**	**(0.9572-0.9906)**	**0.32**	
	F2	1	0.77		(0.647-0.8718)	-0.07		1		(0.9302-0.9995)	-	-
		2	0.71		(0.5826-0.825)	-0.02		1		(0.9302-0.9995)	-	-
		3	0.67		(0.541-0.7924)	-0.40	**	1		(0.9302-0.9995)	-	-
		Total	**0.72**	**0.529**	**(0.6632-0.7646)**	**-0.17**	*	**1**	-	**(0.9878-0.9999)**	-	-
	F3	1	0.6		(0.461-0.724)	0.00		1		(0.9302-0.9995)	-	-
		2	0.64		(0.5006-0.7586)	0.13		1		(0.9302-0.9995)	-	-
		3	0.61		(0.4806-0.7415)	0.03		1		(0.9302-0.9995)	-	-
		Total	**0.617**	**0.919**	**(0.5604-0.6698)**	**0.06**		**1**	-	**(0.9878-0.9999)**	-	-
	F4	1	0.62		(0.4806-0.7415)	-0.02		1		(0.9302-0.9995)	-	-
		2	0.6		(0.461-0.724)	-0.25		1		(0.9302-0.9995)	-	-
		3	0.64		(0.5006-0.7586)	-0.04		1		(0.9302-0.9995)	-	-
		Total	**0.62**	**0.919**	**(0.5638-0.673)**	**-0.10**		**1**	-	**(0.9878-0.9999)**	-	-
	F5	1	0.45		(0.3295-0.5966)	-0.25		0.99		(0.9302-0.9995)	-0.01	
		2	0.62		(0.4806-0.7415)	-0.27		0.98		(0.8953-0.9952)	-0.02	
		3	0.47		(0.3476-0.6156)	-0.41	**	0.99		(0.9302-0.9995)	-0.01	
		Total	**0.51**	**0.218**	**(0.4569-0.5693)**	**-0.28**	***	**0.99**	**0.365**	**(0.9664-0.9946)**	**-0.01**	

(Continued)

Table 4.2S. (Continued)

Site	G	Rep	I1,016	*P*	95% HDI	F_{IS}	Sig	C1,534	*P*	95% HDI	F_{IS}	Sig
	F6	1	0.53		(0.4032-0.6706)	-0.16		1.00		(0.9302-0.9995)	-	-
		2	0.48		(0.3476-0.6156)	0.12		1.00		(0.9302-0.9995)	-	-
		3	0.59		(0.461-0.724)	-0.12		1.00		(0.9302-0.9995)	-	-
		Total	**0.53**	**0.485**	**(0.4768-0.5889)**	**-0.05**		**1.00**	**-**	**(0.9878-0.9999)**	-	-
	F7	1	0.52		(0.3845-0.6522)	0.12		1.00		(0.9302-0.9995)	-	-
		2	0.62		(0.4806-0.7415)	-0.02		1.00		(0.9302-0.9995)	-	-
		3	0.55		(0.4222-0.6885)	0.23		1.00		(0.9302-0.9995)	-	-
		Total	**0.56**	**0.597**	**(0.5067-0.6183)**	**0.12**		**1.00**	**-**	**(0.9878-0.9999)**	-	-
	F8	1	0.59		(0.461-0.724)	0.38	**	1.00		(0.9302-0.9995)	-	-
		2	0.57		(0.4415-0.7062)	0.06		0.99		(0.9302-0.9995)	-0.01	
		3	0.6		(0.461-0.724)	0.00		1.00		(0.9302-0.9995)	-	-
		Total	**0.59**	**0.973**	**(0.5301-0.641)**	**0.15**		**1.00**	**-**	**(0.9816-0.9992)**	**0.00**	
Mer1												
	F1	1	0.59		(0.461-0.724)	-0.03		0.88		(0.7615-0.9428)	0.05	
		2	0.58		(0.4415-0.7062)	-0.23		0.94		(0.8379-0.9782)	-0.06	
		3	0.64		(0.4345-0.8369)	-0.08		0.92		(0.7392-0.987)	-0.09	
		Total	**0.59**	**0.812**	**(0.5295-0.6539)**	**-0.12**		**0.91**	**0.498**	**(0.8678-0.9409)**	**0.01**	
	F2	1	0.43		(0.3114-0.5777)	0.31	*	0.92		(0.8115-0.9673)	-0.09	
		2	0.44		(0.3114-0.5777)	-0.06		0.88		(0.7615-0.9428)	-0.14	
		3	0.48		(0.3476-0.6156)	0.12		0.93		(0.8379-0.9782)	-0.08	
		Total	**0.45**	**0.898**	**(0.3946-0.5066)**	**0.13**		**0.91**	**0.555**	**(0.8722-0.9373)**	**-0.10**	
	F3	1	0.47		(0.3476-0.6156)	0.40	**	0.77		(0.647-0.8718)	-0.30	
		2	0.43		(0.3114-0.5777)	0.06		0.95		(0.8655-0.9877)	0.79	
		3	0.45		(0.3295-0.5966)	0.07		0.95		(0.8655-0.9877)	-0.05	
		Total	**0.45**	**0.923**	**(0.3946-0.5066)**	**0.18**	*	**0.89**	**0.002**	**(0.8495-0.9205)**	**0.01**	
	F4	1	0.3		(0.1913-0.4383)	0.14		0.94		(0.8379-0.9782)	-0.06	
		2	0.47		(0.3476-0.6156)	0.24		0.94		(0.8379-0.9782)	-0.06	
		3	0.38		(0.2586-0.5193)	-0.02		0.94		(0.8379-0.9782)	-0.06	
		Total	**0.38**	**0.180**	**(0.3301-0.4395)**	**0.14**		**0.94**	**1.000**	**(0.9072-0.9615)**	**-0.06**	
	F5	1	0.45		(0.3295-0.5966)	-0.17		1.00		(0.9302-0.9995)	-	-
		2	0.43		(0.3114-0.5777)	-0.02		1.00		(0.9302-0.9995)	-	-
		3	0.52		(0.3845-0.6522)	-0.12		0.97		(0.8953-0.9952)	-0.03	
		Total	**0.47**	**0.706**	**(0.4109-0.5233)**	**-0.10**		**0.99**	**0.365**	**(0.9712-0.9964)**	**-0.01**	
	F6	1	0.49		(0.3658-0.634)	-0.16		0.99		(0.9302-0.9995)	-0.01	
		2	0.46		(0.3295-0.5966)	0.03		0.96		(0.8655-0.9877)	-0.04	
		3	0.53		(0.4032-0.6706)	0.32	*	0.98		(0.8953-0.9952)	-0.02	
		Total	**0.49**	**0.726**	**(0.4372-0.5497)**	**0.07**		**0.98**	**0.360**	**(0.9527-0.9884)**	**-0.02**	
	F7	1	0.46		(0.3295-0.5966)	-0.21		0.95		(0.8655-0.9877)	0.37	
		2	0.32		(0.2077-0.4589)	-0.10		0.94		(0.8379-0.9782)	1.00	
		3	0.41		(0.2937-0.5587)	0.13		0.99		(0.9302-0.9995)	-0.01	
		Total	**0.40**	**0.339**	**(0.343-0.4531)**	**-0.05**		**0.96**	**0.235**	**(0.9315-0.9768)**	**0.65**	
	F8	1	0.23		(0.1435-0.3748)	0.15		0.94		(0.8379-0.9782)	0.29	
		2	0.45		(0.3295-0.5966)	-0.01		0.98		(0.8953-0.9952)	-0.02	
		3	0.35		(0.2414-0.4994)	-0.28		0.97		(0.8953-0.9952)	-0.03	
		Total	**0.34**	**0.070**	**(0.2919-0.3988)**	**-0.02**		**0.96**	**0.437**	**(0.9356-0.9792)**	**0.15**	
Mer2												
	F1	1	0.54		(0.4032-0.6706)	0.20		0.84		(0.7143-0.9157)	0.26	
		2	0.58		(0.4415-0.7062)	0.18		0.85		(0.7377-0.9296)	0.14	
		3	0.64		(0.5006-0.7586)	0.05		0.91		(0.8115-0.9673)	0.15	
		Total	**0.59**	**0.593**	**(0.5301-0.641)**	**0.15**		**0.87**	**0.457**	**(0.8235-0.9005)**	**0.19**	
	F2	1	0.46		(0.3295-0.5966)	0.44	**	0.81		(0.6915-0.9015)	0.03	
		2	0.45		(0.3295-0.5966)	-0.09		0.80		(0.669-0.8869)	0.00	
		3	0.35		(0.2414-0.4994)	-0.19		0.70		(0.5616-0.8088)	0.33	
		Total	**0.42**	**0.506**	**(0.3655-0.4765)**	**0.07**		**0.77**	**0.308**	**(0.7191-0.814)**	**0.15**	

(Continued)

Table 4.2S. (Continued)

Site	G	Rep	I1,016	*P*	95% HDI	F_{IS}	Sig	C1,534	*P*	95% HDI	F_{IS}	Sig
	F3	1	0.43		(0.3114-0.5777)	0.14		0.78		(0.647-0.8718)	-0.05	
		2	0.36		(0.2414-0.4994)	-0.30	*	0.68		(0.541-0.7924)	-0.38	
		3	0.42		(0.2937-0.5587)	0.18		0.73		(0.6037-0.8409)	0.04	
		Total	**0.40**	**0.698**	**(0.3494-0.4598)**	**0.02**		**0.73**	**0.523**	**(0.6771-0.777)**	**-0.13**	
	F4	1	0.38		(0.2586-0.5193)	-0.19		0.79		(0.669-0.8869)	-0.03	
		2	0.4		(0.276-0.5391)	0.33	*	0.77		(0.647-0.8718)	-0.07	
		3	0.33		(0.2245-0.4793)	0.14		0.79		(0.669-0.8869)	-0.15	
		Total	**0.37**	**0.819**	**(0.3173-0.426)**	**0.10**		**0.78**	**0.960**	**(0.733-0.8261)**	**-0.08**	
	F5	1	0.38		(0.2586-0.5193)	-0.02		0.58		(0.4415-0.7062)	-0.07	
		2	0.37		(0.2586-0.5193)	0.10		0.56		(0.4222-0.6885)	0.11	
		3	0.34		(0.2245-0.4793)	-0.25		0.46		(0.3295-0.5966)	0.11	
		Total	**0.36**	**0.892**	**(0.311-0.4193)**	**-0.05**		**0.53**	**0.436**	**(0.4768-0.5889)**	**0.06**	
	F6	1	0.21		(0.1283-0.3531)	-0.03		0.33		(0.2245-0.4793)	0.05	
		2	0.37		(0.2586-0.5193)	0.10		0.52		(0.3845-0.6522)	0.12	
		3	0.3		(0.1913-0.4383)	0.14		0.47		(0.3476-0.6156)	-0.08	
		Total	**0.29**	**0.218**	**(0.2447-0.3472)**	**0.10**		**0.44**	**0.164**	**(0.3849-0.4966)**	**0.05**	
	F7	1	0.31		(0.2077-0.4589)	-0.26		0.45		(0.3295-0.5966)	-0.25	
		2	0.28		(0.175-0.4174)	-0.09		0.49		(0.3658-0.634)	0.08	
		3	0.35		(0.2414-0.4994)	-0.01		0.56		(0.4222-0.6885)	-0.22	
		Total	**0.31**	**0.692**	**(0.2634-0.3679)**	**-0.12**		**0.50**	**0.602**	**(0.4437-0.5562)**	**-0.12**	
	F8	1	0.25		(0.1592-0.3964)	-0.01		0.52		(0.3845-0.6522)	-0.04	
		2	0.37		(0.2586-0.5193)	-0.16		0.49		(0.3658-0.634)	0.08	
		3	0.33		(0.2189-0.4896)	-0.29		0.52		(0.3785-0.6627)	0.00	
		Total	**0.32**	**0.428**	**(0.265-0.3718)**	**-0.14**		**0.51**	**0.971**	**(0.4529-0.5676)**	**0.01**	
Mer3												
	F1	1	0.63		(0.5006-0.7586)	0.01		1.00		(0.9302-0.9995)	-	-
		2	0.81		(0.6915-0.9015)	0.03		1.00		(0.9302-0.9995)	-	-
		3	0.75		(0.6253-0.8565)	-0.23		1.00		(0.9302-0.9995)	-	-
		Total	**0.73**	**0.113**	**(0.6771-0.777)**	**-0.03**		**1.00**	**-**	**(0.9878-0.9999)**	-	-
	F2	1	0.81		(0.6915-0.9015)	0.03		1.00		(0.9302-0.9995)	-	-
		2	0.72		(0.5826-0.825)	0.21		0.98		(0.8953-0.9952)	1.00	
		3	0.78		(0.647-0.8718)	0.07		1.00		(0.9302-0.9995)	-	-
		Total	**0.77**	**0.486**	**(0.7191-0.814)**	**0.12**		**0.99**	**0.365**	**(0.9762-0.9979)**	**1.00**	***
	F3	1	0.63		(0.5006-0.7586)	0.27		0.90		(0.7859-0.9554)	1.00	
		2	0.74		(0.6037-0.8409)	0.38	**	0.98		(0.8953-0.9952)	1.00	
		3	0.8		(0.669-0.8869)	0.00		1.00		(0.9302-0.9995)	-	-
		Total	**0.72**	**0.193**	**(0.6701-0.7708)**	**0.25**	**	**0.96**	**0.026**	**(0.9315-0.9768)**	**1.00**	***
	F4	1	0.83		(0.7143-0.9157)	0.08		1.00		(0.9302-0.9995)	-	-
		2	0.63		(0.5006-0.7586)	0.27		0.99		(0.9302-0.9995)	-0.01	
		3	0.67		(0.541-0.7924)	0.23		0.82		(0.6915-0.9015)	-0.08	
		Total	**0.71**	**0.062**	**(0.6562-0.7583)**	**0.24**	**	**0.94**	**0.000**	**(0.9032-0.9589)**	**0.05**	
	F5	1	0.41		(0.2937-0.5587)	0.05		0.99		(0.9302-0.9995)	-0.01	
		2	0.52		(0.3845-0.6522)	-0.04		1.00		(0.9302-0.9995)	-	-
		3	0.4		(0.276-0.5391)	0.00		1.00		(0.9302-0.9995)	-	-
		Total	**0.44**	**0.433**	**(0.3881-0.5)**	**0.01**		**1.00**	**-**	**(0.9816-0.9992)**	**0.00**	
	F6	1	0.44		(0.3114-0.5777)	0.11		1.00		(0.9302-0.9995)	-	-
		2	0.48		(0.3476-0.6156)	0.20		1.00		(0.9302-0.9995)	-	-
		3	0.46		(0.3295-0.5966)	-0.05		1.00		(0.9302-0.9995)	-	-
		Total	**0.46**	**0.923**	**(0.4044-0.5166)**	**0.09**		**1.00**	**-**	**(0.9878-0.9999)**	-	-
	F7	1	0.38		(0.2586-0.5193)	-0.19		1.00		(0.9302-0.9995)	-	-
		2	0.47		(0.3476-0.6156)	0.08		0.99		(0.9302-0.9995)	-0.01	
		3	0.65		(0.5206-0.7756)	0.17		1.00		(0.9302-0.9995)	-	-
		Total	**0.50**	**0.018**	**(0.4437-0.5562)**	**0.07**		**1.00**	**-**	**(0.9816-0.9992)**	**0.00**	
	F8	1	0.63		(0.5006-0.7586)	-0.24		1.00		(0.9302-0.9995)	-	-

(Continued)

Table 4.2S. (Continued)

Site	G	Rep	I1,016	*P*	95% HDI	F_{IS}	Sig	C1,534	*P*	95% HDI	F_{IS}	Sig
		2	0.63		(0.5006-0.7586)	0.27		0.99		(0.9302-0.9995)	-0.01	
		3	0.64		(0.5006-0.7586)	-0.22		1.00		(0.9302-0.9995)	-	-
		Total	**0.63**	**1.000**	**(0.5773-0.6858)**	**-0.06**		**1.00**	**-**	**(0.9816-0.9992)**	**0.00**	
Dz												
	F1	1	0.37		(0.2586-0.5193)	0.10		0.71		(0.5826-0.825)	0.08	
		2	0.29		(0.1913-0.4383)	0.08		0.62		(0.4806-0.7415)	0.07	
		3	0.39		(0.276-0.5391)	-0.22		0.69		(0.5616-0.8088)	-0.17	
		Total	**0.35**	**0.545**	**(0.2982-0.4056)**	**-0.01**		**0.67**	**0.526**	**(0.6183-0.7239)**	**0.00**	
	F2	1	0.70		(0.5616-0.8088)	-0.14		0.98		(0.8953-0.9952)	1.00	
		2	0.58		(0.4415-0.7062)	-0.23		0.86		(0.7377-0.9296)	0.00	
		3	0.37		(0.2586-0.5193)	-0.24		0.71		(0.5826-0.825)	-0.02	
		Total	**0.55**	**0.005**	**(0.4933-0.6053)**	**-0.12**		**0.85**	**0.001**	**(0.8051-0.8859)**	**0.14**	
	F3	1	0.49		(0.3658-0.634)	0.00		0.67		(0.541-0.7924)	0.05	
		2	0.37		(0.2586-0.5193)	-0.24		0.62		(0.4806-0.7415)	-0.10	
		3	0.40		(0.276-0.5391)	-0.17		0.72		(0.5826-0.825)	-0.29	
		Total	**0.42**	**0.430**	**(0.3655-0.4765)**	**-0.12**		**0.67**	**0.562**	**(0.6149-0.7207)**	**-0.10**	
	F4	1	0.40		(0.276-0.5391)	-0.42	**	0.52		(0.3845-0.6522)	0.52	
		2	0.41		(0.2937-0.5587)	-0.36	*	0.70		(0.5616-0.8088)	0.05	
		3	0.58		(0.4415-0.7062)	-0.31	*	0.77		(0.647-0.8718)	0.15	
		Total	**0.463**	**0.142**	**(0.4077-0.52)**	**-0.33**	***	**0.66**	**0.019**	**(0.608-0.7144)**	**0.30**	
	F5	1	0.42		(0.2937-0.5587)	-0.31	*	0.79		(0.669-0.8869)	-0.27	
		2	0.44		(0.3114-0.5777)	-0.38	**	0.79		(0.669-0.8869)	-0.27	
		3	0.46		(0.3295-0.5966)	-0.05		0.74		(0.6037-0.8409)	-0.35	
		Total	**0.44**	**0.922**	**(0.3849-0.4966)**	**-0.25**	**	**0.77**	**0.705**	**(0.7226-0.817)**	**-0.29**	
	F6	1	0.47		(0.3476-0.6156)	-0.24		0.74		(0.6037-0.8409)	-0.04	
		2	0.48		(0.3476-0.6156)	-0.04		0.68		(0.541-0.7924)	0.08	
		3	0.45		(0.3295-0.5966)	-0.01		0.69		(0.5616-0.8088)	0.02	
		Total	**0.467**	**0.974**	**(0.4109-0.5233)**	**-0.10**		**0.70**	**0.798**	**(0.6493-0.7521)**	**0.03**	
	F7	1	0.36		(0.2414-0.4995)	-0.30	*	0.68		(0.541-0.7924)	-0.01	
		2	0.37		(0.2586-0.5193)	-0.16		0.60		(0.461-0.724)	-0.17	
		3	0.42		(0.2937-0.5587)	-0.15		0.65		(0.5206-0.7756)	-0.10	
		Total	**0.383**	**0.821**	**(0.3301-0.4395)**	**-0.20**	*	**0.64**	**0.684**	**(0.5875-0.6953)**	**-0.09**	
	F8	1	0.30		(0.1913-0.4383)	-0.14		0.42		(0.2937-0.5587)	-0.07	
		2	0.29		(0.1913-0.4383)	-0.21		0.58		(0.4415-0.7062)	0.02	
		3	0.39		(0.276-0.5391)	-0.05		0.74		(0.6037-0.8409)	0.27	
		Total	**0.327**	**0.472**	**(0.2761-0.3816)**	**-0.12**		**0.58**	**0.005**	**(0.5234-0.6345)**	**0.12**	
Co												
	F1	1	0.69		(0.5616-0.8088)	-0.08		0.88		(0.7615-0.9428)	0.43	
		2	0.48		(0.3476-0.6156)	-0.04		0.57		(0.4415-0.7062)	0.47	
		3	0.65		(0.5206-0.7756)	0.08		0.80		(0.669-0.8869)	0.25	
		Total	**0.607**	**0.055**	**(0.5503-0.6602)**	**0.02**		**0.75**	**0.002**	**(0.698-0.7955)**	**0.45**	
	F2	1	0.84		(0.7143-0.9157)	0.11		0.92		(0.8115-0.9673)	0.19	
		2	0.57		(0.4415-0.7062)	-0.10		0.76		(0.6253-0.8565)	-0.10	
		3	0.46		(0.3295-0.5966)	-0.29	*	0.70		(0.5616-0.8088)	-0.24	
		Total	**0.623**	**0.000**	**(0.5672-0.6762)**	**-0.01**		**0.79**	**0.019**	**(0.7439-0.8352)**	**-0.06**	
	F3	1	0.51		(0.3845-0.6522)	0.08		0.63		(0.5006-0.7586)	0.01	
		2	0.44		(0.3114-0.5777)	-0.30	*	0.68		(0.541-0.7924)	-0.10	
		3	0.48		(0.3476-0.6156)	0.12		0.69		(0.5616-0.8088)	0.21	
		Total	**0.477**	**0.726**	**(0.4207-0.5332)**	**-0.03**		**0.67**	**0.809**	**(0.6114-0.7175)**	**0.04**	
	F4	1	0.46		(0.3295-0.5966)	-0.29	*	0.72		(0.5826-0.825)	-0.29	
		2	0.45		(0.3295-0.5966)	-0.50	***	0.65		(0.5206-0.7756)	-0.36	
		3	0.47		(0.3476-0.6156)	0.08		0.59		(0.461-0.724)	0.13	
		Total	**0.46**	**0.974**	**(0.4044-0.5166)**	**-0.24**	**	**0.65**	**0.448**	**(0.5978-0.7049)**	**-0.15**	
	F5	1	0.18		(0.09863-0.3085)	0.19		0.28		(0.175-0.4174)	-0.19	

(Continued)

Table 4.2S. (Continued)

Site	G	Rep	I1,016	*P*	95% HDI	F_{IS}	Sig	C1,534	*P*	95% HDI	F_{IS}	Sig
		2	0.16		(0.08439-0.2857)	0.26		0.30		(0.1913-0.4383)	-0.05	
		3	0.25		(0.1592-0.3964)	0.20		0.38		(0.2586-0.5193)	-0.19	
		Total	**0.197**	**0.417**	**(0.1557-0.2454)**	**0.22**	**	**0.32**	**0.526**	**(0.2698-0.3748)**	**-0.13**	
	F6	1	0.06		(0.02176-0.1621)	-0.06		0.27		(0.175-0.4174)	0.24	
		2	0.07		(0.03267-0.1885)	-0.08		0.28		(0.175-0.4174)	-0.19	
		3	0.09		(0.0446-0.2138)	-0.10		0.26		(0.1592-0.3964)	0.17	
		Total	**0.073**	**0.762**	**(0.04908-0.1086)**	**-0.08**		**0.27**	**0.967**	**(0.2229-0.3229)**	**0.07**	
	F7	1	0.18		(0.09863-0.3085)	-0.08		0.41		(0.2937-0.5587)	-0.12	
		2	0.1		(0.0446-0.2138)	0.11		0.18		(0.09863-0.3085)	0.05	
		3	0.08		(0.03267-0.1885)	-0.09		0.12		(0.05727-0.2383)	-0.14	
		Total	**0.12**	**0.266**	**(0.08801-0.1617)**	**-0.01**		**0.24**	**0.001**	**(0.1921-0.2879)**	**0.02**	
	F8	1	0.6		(0.461-0.724)	-0.08		0.16		(0.08439-0.2857)	0.26	
		2	0.31		(0.2077-0.4589)	-0.26		0.09		(0.0446-0.2138)	-0.10	
		3	0.37		(0.2586-0.5193)	0.01		0.62		(0.4806-0.7415)	0.15	
		Total	**0.427**	**0.012**	**(0.3719-0.4832)**	**-0.04**		**0.29**	**0.000**	**(0.2416-0.3439)**	**0.37**	
Ac												
	F1	1	0.79		(0.6532-0.8744)	0.22		0.94		(0.8379-0.9782)	-0.06	
		2	0.78		(0.647-0.8718)	0.07		0.92		(0.8115-0.9673)	0.19	
		3	0.74		(0.6037-0.8409)	0.06		0.89		(0.7859-0.9554)	0.29	
		Total	**0.77**	**0.766**	**(0.7191-0.814)**	**0.12**		**0.92**	**0.762**	**(0.8799-0.9428)**	**0.17**	
	F2	1	0.54		(0.4032-0.6706)	-0.21		0.66		(0.5206-0.7756)	0.11	
		2	0.74		(0.6037-0.8409)	-0.25		0.90		(0.7859-0.9554)	0.11	
		3	0.80		(0.6532-0.8744)	0.00		0.91		(0.8115-0.9673)	0.15	
		Total	**0.693**	**0.013**	**(0.6389-0.7427)**	**-0.10**		**0.82**	**0.001**	**(0.7761-0.8623)**	**0.20**	
	F3	1	0.55		(0.4222-0.6885)	-0.01		0.71		(0.5826-0.825)	0.17	
		2	0.74		(0.6037-0.8409)	-0.04		0.85		(0.7377-0.9296)	-0.18	
		3	0.74		(0.6037-0.8409)	-0.14		0.90		(0.7859-0.9554)	0.11	
		Total	**0.677**	**0.084**	**(0.6217-0.727)**	**-0.02**		**0.82**	**0.044**	**(0.7725-0.8593)**	**0.10**	
	F4	1	0.60		(0.461-0.724)	-0.25		0.81		(0.6915-0.9015)	-0.24	
		2	0.64		(0.5006-0.7586)	-0.22		0.86		(0.7377-0.9296)	-0.16	
		3	0.56		(0.4222-0.6885)	-0.22		0.83		(0.7143-0.9157)	0.22	
		Total	**0.6**	**0.717**	**(0.5435-0.6538)**	**-0.22**	**	**0.83**	**0.862**	**(0.7869-0.8712)**	**-0.06**	
	F5	1	0.58		(0.4415-0.7062)	-0.15		0.93		(0.8379-0.9782)	-0.08	
		2	0.58		(0.4415-0.7062)	-0.23		0.94		(0.8379-0.9782)	-0.06	
		3	0.54		(0.4032-0.6706)	-0.21		0.95		(0.8655-0.9877)	-0.05	
		Total	**0.567**	**0.897**	**(0.51-0.6215)**	**-0.20**	*	**0.94**	**0.876**	**(0.9072-0.9615)**	**-0.06**	
	F6	1	0.55		(0.4222-0.6885)	-0.01		0.87		(0.7615-0.9428)	0.03	
		2	0.59		(0.461-0.724)	-0.28	*	0.88		(0.7615-0.9428)	-0.14	
		3	0.61		(0.4806-0.7415)	0.20		0.90		(0.7859-0.9554)	-0.11	
		Total	**0.583**	**0.824**	**(0.5268-0.6378)**	**-0.03**		**0.88**	**0.936**	**(0.842-0.9148)**	**-0.07**	
	F7	1	0.42		(0.2937-0.5587)	-0.07		0.79		(0.669-0.8869)	-0.15	
		2	0.49		(0.3658-0.634)	0.00		0.78		(0.647-0.8718)	-0.17	
		3	0.50		(0.3658-0.634)	0.12		0.77		(0.647-0.8718)	-0.19	
		Total	**0.47**	**0.652**	**(0.4142-0.5265)**	**0.02**		**0.78**	**0.961**	**(0.7297-0.8231)**	**-0.17**	
	F8	1	0.44		(0.3114-0.5777)	0.11		0.77		(0.647-0.8718)	-0.07	
		2	0.41		(0.2937-0.5587)	-0.28	*	0.80		(0.669-0.8869)	-0.13	
		3	0.41		(0.2937-0.5587)	-0.28	*	0.75		(0.6253-0.8565)	-0.12	
		Total	**0.42**	**0.973**	**(0.3655-0.4765)**	**-0.15**		**0.77**	**0.890**	**(0.7226-0.817)**	**-0.10**	

G=Generation, *P*= chi-square probability value of, HDI = 95% Highest Density Intervals (HDI) around frequencies and F_{IS} = Inbreeding coefficients in eight generations of pyrethroid relaxation in eight Southern Mexican *Aedes aegypti*.

Table 4.3S. Permethrin LC_{50} in *Ae. aegypti* generations F3, F6 and F8 from eight collection sites from Southern Mexico. Sample size (N), standard error (SE), 95% confident interval (95% CI), data adjusted to logistic model *p*-value > 0.05.

						95% CI		
Site	**Generation**	**N**	**Slope**	**SE**	**LC_{50}**	**Lower**	**Upper**	***p*-value**
New Orleans								
	F3	294	1.9110	0.2707	**0.58**	0.49	0.68	0.011385
	F6	429	2.3609	0.2614	**0.64**	0.58	0.71	0.000761
	F8	523	1.9425	0.1882	**0.49**	0.44	0.55	**0.405565**
Acp								
	F3	818	1.2374	0.1110	**28.95**	24.57	34.11	2.55E-09
	F6	498	1.2010	0.1508	**16.37**	13.98	19.17	2.96E-13
	F8	499	2.1370	0.1965	**5.30**	4.58	6.13	0.023662
Tap								
	F3	703	1.2032	0.1538	**19.81**	17.39	22.56	1.81E-11
	F6	412	1.2563	0.1897	**29.08**	24.03	35.19	3.81E-06
	F8	921	2.1079	0.1385	**15.61**	14.41	16.92	0.012831
Mer1								
	F3	450	1.6087	0.1861	**21.50**	17.38	26.59	6.71E-14
	F6	536	2.6153	0.4817	**15.08**	11.55	19.69	**0.325776**
	F8	431	5.7311	0.6370	**10.65**	10.10	11.23	0.020522
Mer2								
	F3	-	-	-	-			-
	F6	474	1.3569	0.1134	**10.38**	8.70	12.39	**0.397436**
	F8	600	1.8243	0.1364	**5.62**	**4.92**	**6.42**	8.85E-13
Mer3								
	F3	534	2.1211	0.1763	**25.45**	23.03	28.11	**0.54884**
	F6	834	1.3841	0.1022	**31.26**	27.47	35.57	0.01185
	F8	357	1.3794	0.1483	**1.85**	1.52	2.25	**0.324171**
Dz								
	F3	471	0.9300	0.0954	**12.78**	9.77	16.73	5.84E-05
	F6	465	0.8085	0.0893	**19.36**	14.95	25.07	2.09E-23
	F8	368	1.3129	0.2972	**9.79**	7.18	13.34	**0.857938**
Co								
	F3	385	1.2687	0.1562	**9.63**	8.08	11.49	**0.949819**
	F6	549	1.0742	0.1439	**1.33**	0.86	2.04	3.66E-40
	F8	877	1.3149	0.0845	**1.33**	1.15	1.53	6.64E-22
Ac								
	F3	-	-	-	-			-
	F6	700	1.7505	0.1794	**24.70**	21.61	28.24	**0.316734**
	F8	594	3.0739	0.2665	**17.76**	16.60	18.99	8.36E-27

Table 4.4S. Deltamethrin LC_{50} in *Ae. aegypti* generations F3, F6 and F8 from eight collection sites from Southern Mexico. Sample size (N), standard error (SE), 95% confident interval (95% CI), data adjusted to logistic model *p*-value > 0.05.

						95% CL		
Site	**Generation**	**N**	**Slope**	**SE**	**LC50**	**Lower**	**Upper**	***p*-value**
New Orleans								
	F3	412	7.2728	1.1197	**0.09**	0.09	0.10	0
	F6	336	2.4218	0.3029	**0.12**	0.10	0.14	**0.267636**
	F8	479	2.1420	0.1864	**0.21**	0.18	0.24	4.77E-34
Acp								
	F3	731	2.8688	0.2386	**7.66**	7.12	8.24	4.87E-06
	F6	316	1.5934	0.1706	**2.27**	1.86	2.76	**0.532967**
	F8	530	0.6918	0.0877	**0.44**	0.32	0.61	**0.6555**
Tap								
	F3	703	1.2032	0.1538	**8.88**	8.29	9.51	1.81E-11
	F6	819	2.6528	0.1624	**2.32**	2.14	2.52	1.22E-35
	F8	915	1.2145	0.0917	**4.26**	3.76	4.83	0.037817
Mer1								
	F3	-	-	-	**-**	-	-	-
	F6	705	1.9391	0.2559	**1.31**	1.08	1.58	0.034772
	F8	289	4.3418	0.6354	**2.29**	2.12	2.47	8.23E-11
Mer2								
	F3	-	-	-	-	-	-	-
	F6	684	0.7725	0.0869	**0.41**	0.32	0.51	9.7E-11
	F8	394	1.1749	0.1225	**0.44**	**0.36**	**0.53**	1.12E-16
Mer3								
	F3	-	-	-	**-**	-	-	**-**
	F6	786	0.6870	0.0882	**1.16**	0.93	1.43	2.78E-12
	F8	261	1.7308	0.2387	**0.04**	0.03	0.05	**0.417359**
Dz								
	F3	372	0.4458	0.1517	**0.52**	0.08	3.42	0.003115
	F6	954	1.1296	0.0911	**0.81**	0.71	0.92	1.9E-92
	F8	568	0.3405	0.1087	**7.73**	1.62	36.88	**0.98442**
Co								
	F3	385	1.2687	0.1562	**9.63**	8.08	11.49	**0.949819**
	F6	477	2.9720	0.2819	**0.65**	0.60	0.71	1.03E-48
	F8	510	1.4261	0.0000	**0.25**	0.20	0.32	2.82E-05
Ac								
	F3	423	1.3751	0.1445	2.85	2.25	3.62	**0.665849**
	F6	828	1.5002	0.1123	**3.21**	2.79	3.68	1.09E-23
	F8	304	0.7999	0.1285	**1.82**	1.34	2.47	**0.242328**