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Ecological mediators of pyrethroid resistance evolution in *Aedes aegypti*

By

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Population Biology, Ecology, and Evolution

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Ecological mediators of pyrethroid resistance evolution in *Aedes aegypti*

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An abstract of
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Abstract

Chemical insecticides are used globally to prevent the transmission of vector-borne pathogens to humans. Due to the strong selective pressures insecticides exert on target vector populations, insecticide resistance has evolved in all major vector species. Resistance is of particular concern for the prevention of dengue, chikungunya, and Zika, diseases that are transmitted by the *Aedes aegypti* mosquito, since insecticides are currently the only way to prevent and curtail outbreaks. One prevalent form of resistance in *Ae. aegypti*, called "knock-down resistance" (*kdr)*, confers resistance to pyrethroid insecticides through point mutations in the sodium channel gene. Research on insecticide resistance in *Ae. aegypti* has largely focused on determining the underlying genes and molecular mechanisms responsible for resistance, and has mostly overlooked the ecological processes that mediate the evolution of resistance. For example, little is known about how resistance genes spread within and between populations, and whether there exists a fitness cost to resistance. These knowledge gaps limit our ability to effectively manage resistance at the population level. The objective of my dissertation is to understand some of the ecological processes underlying resistance evolution in natural populations of *Ae. aegypti*. Chapter 1 describes the spatial and temporal patterns of *kdr* allele frequencies in a small city in the Yucatán, Mexico, and shows that allele frequencies are highly heterogeneous between city blocks, a spatial scale that had not been previously assessed. Furthermore, *kdr* allele frequencies were significantly lower after the dry season, suggesting that there may be a fitness cost to pyrethroid resistance in the absence of strong insecticide pressure. Chapter 2 provides empirical evidence of a fitness cost to pyrethroid resistance in *Ae. aegypti*, measured through various life history traits, and shows that density-dependent intraspecific competition can mediate resistance phenotype and genotype. Chapter 3 provides further evidence of a fitness cost to resistance and shows that pyrethroid susceptibility can be restored in just over 10 generations without insecticide pressure. These results lend support to vector control strategies that vary insecticide application in time and/or space, leveraging the fitness cost of resistance in the absence of insecticides to regain susceptibility in populations.

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Introduction

Insecticide resistance is an increasing challenge in the control of vector-borne diseases. The majority of vector control programs rely heavily on insecticide-based interventions, such as the use of long-lasting insecticide-treated bed nets (LLITN), indoor residual spraying (IRS), ultra-low volume spraying (ULV) and larviciding, creating multiple selective pressures on vector populations (1-3). Currently, all major vector species have developed resistance to at least one class of insecticide (4).

There are four main mechanisms of insecticide resistance: (1) target site resistance, where a point mutation in a structural gene reduces the sensitivity of the neuronal channels to the insecticide, (2) metabolic resistance, which is commonly an overexpression of detoxification enzymes, (3) reduced penetration of the insecticide across the cuticle, and (4) behavioral resistance, which is the ability of the insect to detect and avoid the insecticide (5, 6). Target site resistance is the most common mechanism, and several species have gained a particular form known as "knock-down resistance," or *kdr* (6, 7). *Kdr* are point mutations in the sodium channel gene that reduce the ability of pyrethroid insecticides to bind to the voltage-gated sodium channels found in nerve cell membranes of insects (6). Pyrethroid insecticides are still the only class of insecticide approved for use in insecticide-treated materials due to their low mammalian toxicity (4), so a firm understanding of *kdr* mechanisms is crucial for effective vector control.

Resistance is of particular concern for controlling *Aedes aegypti*, the main vector of dengue, chikungunya, and Zika, because there is no antiviral treatment and currently no widespread vaccine available for these viruses, making vector control the only way to prevent disease (8, 9). *Ae. aegypti* are highly anthropophilic day-biting mosquitoes that

live in urban areas in and around houses and breed primarily in man-made containers such as buckets or flower pots, making them very difficult to control (10). Unfortunately, vector control measures in general have largely failed in regions where dengue is endemic (9), and *Ae. aegypti* has developed resistance in most locations. The most prevalent form of resistance in *Ae. aegypti* is *kdr* (1). Many *kdr* point mutations have been found in *Ae. aegypti*, though the phenylalanine to cysteine mutation in codon 1534 and the valine to isoleucine in 1016 are most strongly associated with pyrethroid resistance (1).

To date, insecticide resistance studies in disease vectors have mainly focused on the evolutionary mechanisms of resistance: the genes responsible, the physiologic mechanisms underlying resistance, and how different control methods can potentially delay the emergence of resistance (6, 11-13). What is surprisingly absent from the literature are studies that address ecological processes that impact the evolution of resistance. How resistance genes spread within and between populations, how landscape heterogeneity can impact the emergence of resistance, and the spatial scale at which these processes operate are significant knowledge gaps that limit our ability to effectively manage resistance at the population level. For example, two strategies that are commonly used to mitigate evolution of resistance in insects involve rotation of chemicals or mosaic spraying, both of which create a heterogeneous environment in time and/or space (11). The assumption of these strategies is that if resistance develops to an insecticide and then the application of that insecticide halts, susceptibility can be restored into populations. However, that requires a fitness cost to resistance as well as an understanding of how

insect populations interact in time and space, which are mechanisms that are understudied, especially in disease vectors.

Using *Ae. aegypti* as a study organism, I aim to understand the ecological mechanisms underlying the maintenance and propagation of insecticide resistance in patchy environments. My research is conducted in the Yucatan, Mexico, which has experienced a rapid increase in the frequency of the *kdr* mutations in *Ae. aegypti* in the past 20 years along with the rest of the country (14). Beginning in 1947, the Yucatan developed a sophisticated vector control program relying primarily on the use of pyrethroid insecticides (15). This created a strong selection pressure for resistance evolution that has a substantial level of spatial and temporal heterogeneity. Insecticide applications occur in response to the detection of locally-acquired dengue cases (3), creating a mosaic of selection pressure in both space and time, within and between towns. Given that *Ae. aegypti* has a limited flight dispersal ability (most mosquitoes seldom disperse beyond 100m) (16, 17), I have strong evidence to hypothesize that the propagation of *kdr* mutations across the landscape by vector dispersal will be moderate, leading to a tractable system for understanding fine-scale dynamics of resistance evolution.

Specifically, I aim to:

1. Describe the spatial and temporal patterns of insecticide resistance in a satellite town of Mérida, Mexico and determine the spatial scale that best captures these patterns.
2. Determine if there is a fitness cost to resistance and if intraspecific competition mediates the fitness cost.

3. Evaluate the potential to restore susceptibility in a highly resistant strain of *Ae. aegypti* in the absence of insecticide.

I hypothesize that: a) insecticide resistance will be both temporally and spatially heterogeneous, and that fine-scale population dynamics are responsible for the maintenance and propagation of resistance; b) intraspecific and inter-strain competition, between susceptible and resistant individuals, could mediate evolution of insecticide resistance within populations; and c) susceptibility may be restored given a fitness cost to resistance in the absence of insecticide pressure. To test these hypotheses, I will link field observations and experimental studies performed in the Yucatán State, Mexico.

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Chapter 1: Fine-scale spatial and temporal dynamics of knock-down resistance evolution in *Aedes aeygpti*

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Introduction

The recent introduction of Zika and chikunguyna into the Americas, along with the persistence of dengue, has made *Aedes aeygpti* one of the most important mosquito vectors worldwide (1). *Ae. aegypti* are highly anthropophilic day-biting mosquitoes that live in close association with humans, primarily in urban areas. They live and breed in and around houses, with immature stages developing in most water-holding containers, making them very difficult to control (2). *Ae. aegypti* control programs employ a combination of methods including ultra-low volume spraying (ULV), indoor space spraying (ISS), and source reduction through larviciding (3-5). While such efforts have shown isolated successes, most vector control measures have largely failed in regions where dengue is endemic (6), and *Ae. aegypti* has developed resistance to the primary insecticide classes in many locations throughout the world (3).

Historically, the use of pyrethroid insecticides for vector control has been widespread due to their low cost and low mammalian toxicity (7), and consequently, pyrethroid resistance has developed in most medically important vectors (8, 9). In *Ae. aegypti*, pyrethroid resistance occurs through mechanisms such as target-site resistance, where a mutation in a structural gene reduces the sensitivity of the neuronal channels to the insecticide, and metabolic resistance, which is commonly an overexpression of enzymes that metabolize foreign chemicals (7). Point mutations in the para-orthologous

sodium channel gene are a prevalent source of target-site resistance in *Ae. aegypti*, and a well-studied and commonly used indicator of pyrethroid resistance in field populations. Termed "knock-down resistance, or *kdr*, these mutations allow the mosquito to maintain normal function in the presence of a pyrethroid by reducing the ability of the insecticide to bind to the sodium channel (7). Many different *kdr* mutations have been identified, but here we focus on two mutations that have a documented role in pyrethroid resistance in *Ae. aegypti* from Mexico, the valine to isoleucine mutation in the 1016 codon and the phenylalanine to cysteine mutation in 1534 (10-12).

The frequency of *kdr* mutations has been increasing rapidly in time and space throughout the world. For example, in Mexico, the frequency of I1016 increased from <0.1% in 1996-2000 to 88.3% in 2007-2009 (10). Brazil has seen a similar increase in both the C1534 and I1016 *kdr* mutations: in 2002 there was no *kdr* present, yet by 2006 the two *kdr* mutations were detected at low frequencies, and by 2012 the double mutant had the highest frequency (13). Not only are *kdr* mutations increasing in locations where they are already present, but they are evolving in new locations. For example, the first report of *kdr* in Indian *Ae. aegpyti* populations was in 2015 (14); as of 2001, populations in Delhi had been 100% susceptible to pyrethroids (15). However, the most recent reports indicate only 35% mortality to DDT and a 0.41–0.79 frequency of the C1534 *kdr* allele (14). Given these increases, it has become increasingly important to understand the mechanisms underlying *kdr* spread and persistence in field populations.

Previous studies on *kdr* in field populations have largely extrapolated regional patterns of resistance from sampling relatively few locations in a country (13, 14, 16), or they offer a snapshot of resistance patterns through cross-sectional studies that draw a

sample of *kdr* frequencies taken at a single time point during the year (17-19). These sampling schemes may be appropriate given that *Ae. aegypti* populations have been shown to be panmictic at coarser spatial scales (20), suggesting that dispersal is high enough to create a homogeneous population.

One hypothesis is that the reliance of area-wide truck-mounted ULV applications would lead to homogeneous distribution of resistance within larger geographic areas (e.g. neighborhoods). Conversely, the implementation of ISS for the control of *Ae. aegypti* in response to symptomatic disease cases may lead to a heterogeneous pattern, driven by the extent and frequency of insecticide applications (21). Given the reactive nature of dengue control, and the potential for strong variability in insecticide selection pressure in space and time, there is a need to understand the spatial scale of insecticide resistance evolution. The limited flight range of *Ae. aegypti* (of approximately 150m (22)) further complicates predictions of the geographic spread of resistance, providing a justification for the need to study mechanisms of resistance from a spatial and temporal scale that reflects the fine scale at which selection pressure and mosquito dispersal operate.

Here, we aim to assess the spatial and temporal scales that best capture patterns of *kdr* in field populations. A recent study by Deming et al (11) found significant heterogeneity in *kdr* frequencies between interconnected towns in the Yucatan State, Mexico, with only some towns displaying heterogeneity between city blocks. Building from that research, we conducted a more comprehensive sampling of *kdr* in one of the study towns over two years, aiming to answer two questions: (1) are *kdr* frequencies heterogeneous at the fine spatial scale of city blocks, or are they better assessed within city neighborhood, and (2) do *kdr* frequencies vary over a relatively short timeframe,

namely between the wet and dry season within a year. Understanding the scale of *kdr* evolution is not only important for monitoring susceptibility, but also for creating interventions to mitigate resistance evolution while controlling populations.

Methods

Study area

This study was conducted in Hunucma (population approximately 25,000), a small satellite town of Merida, the capital of the Yucatan state, Mexico (Figure 1.1). Hunucma is endemic for the dengue virus, with 771 cases reported since 2008, and there have been 15 reports of chikungunya and 125 cases of Zika beginning in 2016 (Secretary of Health, Yucatan).

A vector control program began in Mexico in 1950, which includes ultra-low volume spraying (ULV), indoor space spraying (ISS), and larviciding. By the year 2000, the program began using permethrin-based insecticides, as mandated by the government, and continued until 2009 (10, 23). Currently in Hunucma, the insecticides malathion, chloropyriphos, bendiocarb, and deltamethrin are all used for adult *Ae. aegypti* control (Yucatan Secretary of Health). Previous research indicates that in Hunucma in 2013, the frequency of the C1534 *kdr* mutation in *Ae. aegypti* was approximately 0.563 and I1016 was 0.586 (11).

Insecticide use data

The Yucatan's Secretary of Health provided information from their Entomological Surveillance and Vector Control Database (24) about the *Ae. aegypti* control efforts in Hunucma during the duration of the study. These data included the type

of insecticide, mode of application (ULV, ISS, or larvicide), and location of application at the city block level.

Entomological collection

We conducted a longitudinal entomological survey twice per year from June 2014-January 2016 at the beginning of the wet season (June-July) and at the beginning of the dry season (January) each year, which corresponds to before and after the dengue transmission season (Figure 1.2). Hunucma has two main roads that run northeast-southwest and northwest-southeast, dividing the city into four sectors. *Ae. aegypti* have been shown to be highly clustered in space (25) with dispersal limited by the urban landscape, such as roads (26), so we chose these four natural sectors as our coarse-scale sampling unit. Within the three smaller sectors, we chose five blocks at random to sample for fine-scale dynamics, and in the larger sector we chose nine blocks at random for greater area coverage, totally 24 blocks. Power calculations for the test of two proportions with alpha=0.025 (two-sided hypothesis) and a beta=0.2 (80% power) indicated that approximately 30 mosquitoes per block were needed to detect a 25% difference in allele frequencies per block. Pilot data estimated 3-5 mosquitoes per house, so we chose to sample at least 10 houses per block, or enough houses until we obtained a sample of at least 30 individuals.

We used a Prokopack aspirator (27) to collect adults from each house for 10 minutes or until the entire house was complete. All collected mosquitoes were transported in plastic cups to the laboratory, where they were placed in a -20ºC freezer for euthanization. We identified each mosquito to species and sex, and only *Ae. aegypti* were

stored in RNA*later®* Stablization Solution (Thermo Fisher Scientific) for *kdr* genotyping.

During the dry season collections in January of each year, there were a low abundance of adult mosquitoes inside homes. To augment sampling efforts, we placed oviposition traps outside four houses per block to collect *Ae. aegypti* eggs, which were then aggregated by block and hatched in the insectary. Thirty-five to fifty adults per block were selected at random for subsequent *kdr* genotyping.

Molecular assays

DNA was extracted from each individual mosquito by using a 50ul solution containing 5ul of Taq 10X buffer (containing 500mM KCl, 100mM tris HCl, 15mM MgCl2, and 1% Triton X-100) and 45ul of sterile ddH_2O and heating in an Eppendorf Mastercycler© pro thermocycler at 95ºC for 15 minutes. Allele-specific real time PCR was conducted using a Biorad© CFX96 machine (Hercules, CA). **1016:** Each 20uL reaction consisted of 8 uL PerfeCTa® SYBR® Green FastMix (Quanta Biosciences), 8.86uL of ddH2O, 0.34uL of 10uM Val1016 forward primer, 0.4 uL of the 10uM Iso1016 forward primer, 0.4uL of the 10uM Iso1016 reverse primer, and 2uL of template DNA (primer sequences below in Table 3). Cycling conditions were 95ºC for 3 min, followed by 35 cycles of 95ºC 10 sec, 60ºC 30 sec, 72ºC 30 sec, and finishing with an incremental temperature increase of 0.2ºC/10s from 65-95ºC to determine the melting point. **1534:** Each 20uL reaction consisted of 9 uL PerfeCTa® SYBR® Green FastMix (Quanta Biosciences), 7.15uL of ddH2O, 0.65uL of 10uM Cys1534 forward primer, 0.60 uL of the 10uM Phe1534 forward primer, 0.60uL of the 10uM Cys1534 reverse primer,

and 2uL of template DNA (primer sequences in Table S1). Cycling conditions were 95ºC for 3 min, followed by 37 cycles of 95ºC 10 sec, 57ºC 30 sec, 72ºC 30 sec, and finishing with an incremental temperature increase of 0.5ºC/5s from 65-95ºC to determine the melting point. Melting curve analysis determined the genotype of the mosquito at each codon; for a complete description of this analysis, see Saavedra-Rodriguez et al (28) for 1016 and Yanola et al (29) for 1534.

Data analysis

Allele frequencies for both the C1534 and I1016 *kdr* mutations were mapped using QGIS 2.18 (QGIS Development Team, 2016) at both the block and sector level for each sampling timepoint. Sampling timepoints in July were characterized as the wet season and timepoints in January were defined as dry. We tested for linkage disequilibrium between the two markers by using the equations outlined in Gillespie (30) to calculate the coefficient D, r^2, and the Chi-square statistic with one degree of freedom. Even though the mutations were in linkage disequilibrium (D=0.137, r^2=0.31, Chi-square=541.9 $p<0.0001$), we chose to analyze the two mutations separately because (1) some blocks displayed significant differences in one mutation over time but not the other, and (2) the C1534 mutation was more variable in time than the I1016 mutation, so we did not want to obscure those findings. Nevertheless, we conducted select analyses on haplotype frequencies.

We used a multiple proportions test with a Chi-square distribution to test for differences in allele frequencies over time for each city block and sector, and for differences between blocks and sectors at each timepoint. The presence of spatial autocorrelation between block-level *kdr* frequencies was assessed with Moran's I statistic

using an inverse distance weighted scheme in the R package ape (31). Moran's I is similar to Pearson's r correlation, yet it tests correlation of values in space. To test for an overall effect of time on block-level allele frequencies, we used a linear mixed model with timepoint as the predictor and block as the random intercept using the R package nlme (32). Additionally, we tested for an effect of season on block-level allele frequencies, aggregating the two dry and two wet season collections together. For this analysis, we also used a linear mixed model with block as a random intercept and season as the only fixed effect. Lastly, we assessed a relationship between the mean number of adult mosquitoes collected in the household per block and the allele frequencies per block. We only used data from the wet season for this analysis since the number of mosquitoes collected inside houses during the dry season was limited. Again, we used a linear mixed model with block as a random intercept.

Ethics Statement

All study protocols were approved by Emory University Institutional Review Board (IRB00082848) as well as the ethics board at the Autonomous University of Yucatan. Written informed consent was obtained from the household owner and houses who did not consent to the study were noted and not surveyed.

Results

Insecticide application

Insecticide applications were highly heterogeneous in space. In 2014 and 2015, the insecticides malathion and chlorpirifos were used for ULV spraying in the study area. Both deltamethrin (a pyrethroid insecticide) and bendiocarb (a carbamate) were used for ISS, though only 6 out of the 24 study blocks (25%) were sprayed at some point during

the study period, so we could not assess any relationship between ISS and *kdr* frequencies (Figure 1.3).

Entomological surveys

We sampled 571 houses from June 2014-Feb 2016, collecting a total of 3,714 *Ae. aegypti*, 63% of which were female (Table 1.1). The mean number of *Ae. aegypti* adult mosquitoes collected per house was 3.5, so data were not analyzed at the household level, only at the block and sector level to ensure sufficient sample size. The mean (±SD) number of indoor resting adult *Ae. aegypti* per block for each of the four time-points was 39.5 (±22.1) in the wet season in 2014, 9.1(±10.9) in dry of 2015, 17.1(±15.6) in wet season of 2015, and 2.2 (±3.1) in the dry season of 2016 (Figure 1.4). If 30 individuals were not caught in a block at a timepoint, we used adults reared from eggs collected in that block as previously described. Total sample sizes for each block at each timepoint can be found in Table S1.2 and Table S1.3.

Kdr frequencies in space

The frequencies of C1534 (Figure 1.5), I1016 (Figure 1.6), and the C1534/I1016 haplotype (Figure S1.1) varied greatly at the block-level throughout the course of the two-year study period. At all timepoints, the frequencies of C1534 were significantly different between blocks (wet 2014: X-squared = 65.8, df=23, p=<0.001; dry 2015: X-squared = 162.9, df = 22, p-value = <0.001; wet 2015: X-squared = 92.9, df = 23, p-value = <0.001; dry 2016: X-squared = 123.5, df = 21, p-value = <0.001). Similarly, frequencies of I1016 were different between blocks at all timepoints (wet 2014: X-squared = 59.7, df = 23, p-value = <0.001; dry 2015: X-squared = 145.7, df = 22, p-value

= <0.001; wet 2015: X-squared=37.1, df = 23, p-value = 0.032; dry 2016: X-squared = 113.8, df = 21, p-value = <0.001). Only two time periods showed evidence of significant autocorrelation in the block-level allele frequencies: the dry season of 2015 for C1534 (I=0.051, p=0.011) and the following wet season of 2015 for 1016 (I=0.059, p=0.003). The positive value of Moran's I indicates that similar values of *kdr* frequencies occurred near each other more often than one would expect by chance. The remaining sampling periods did not have statistical evidence of spatial autocorrelation (I < |0.05|, P>0.05), indicating that *kdr* frequencies were not significantly clustered in space.

When analyzed at the sector level, allele frequencies became more homogeneous (Figure 1.7 & Figure 1.8). There were significant differences in C1534 frequencies between sectors for all timepoints except for the wet season in 2014 (Table S1.4; dry 2015: X-squared = 59.2, df = 3, p-value = <0.001; wet 2015: X-squared = 22.1, df = 3, p-value = <0.001; dry 2016: X-squared = 42.2, df = 3, p-value = <0.001). However, I1016 frequencies only showed differences between blocks during the dry season timepoints (dry 2015: X-squared = 29.2, df = 3, p-value = <0.001; dry 2016: X-squared = 42.1, df = 3, p-value = <0.001).

Kdr frequencies over time

The block-level frequencies of C1534 and I1016 also significantly changed over time (Figure 1.9). Taking the C1534 allele frequency at the first sampling point (wet 2014) as the reference with the null hypothesis of no change over time, there was a significant increase in the frequency of 0.13±0.04 during the dry season directly after in 2015 (Table S1.5; Linear mixed-effects model, t=3.32, p=0.0014). Similarly, there was a

significant increase of 0.08±0.04 (±SE) in C1534 during the dry season of 2016 (Table S1.5; linear mixed-effects model time effect, t=2.18, p=0.032). For I1016, the difference in time was an increase in frequency of 0.09±0.04 between the first wet season collection in 2014, which was the reference, and the subsequent dry season collection in 2015 (Table S1.6; linear mixed-effects model time effect, t=2.37, p=0.021). The frequencies over time for the C1534/I1016 haplotype mirrored that of I1016 (Figure 1.9c), showing a significant increase of 0.09±0.04 between the wet season of 2014 and the dry season of 2015 (Table S1.7; linear mixed effects model time effect, t=2.79, p=0.007).

Aggregating by sector obscured the differences in allele frequencies over time (Figure 1.10). The only significant difference found was a 0.14±0.05 increase in C1534 between the first wet season in 2014 and the following dry season in 2015 (Table S1.8; linear mixed-effects model time effect, t=2.91; p=0.017).

Block-level C1534 frequencies decreased by 0.07±0.03 during the wet season collections compared to the dry season (Table S1.9; linear mixed-effects model time effect, t = -2.55, p = 0.013). The same pattern was noted at the sector level (Table S1.10; linear mixed-effects model time effect, t= -2.88, p = 0.015). Frequencies in I1016 did not show a seasonal effect at either the block level (Table S1.11; linear mixed-effects model time effect, t = - 0.222, p=0.825) or the sector level (Table S1.12; linear mixed-effects model time effect, t = -1.17, p=0.267). Analyzing each block individually for differences in allele frequencies over time, 18 of the 24 blocks (75%) for C1534 were significantly different between time-points (Table S1.2), and 13 of the blocks (54%) for I1016 (Table S1.3). Additionally, there was no significant association between the number of adult mosquitoes collected per block during the wet season in either C1534 (linear mixed-

effects model time effect, t = - 0.73, p=0.467) or I1016 (linear mixed-effects model time effect, t = -1.32, p=0.199).

Discussion

We found significant heterogeneity in the frequency of *kdr* mutations between city blocks, suggesting that *kdr* evolution is occurring at a fine spatial scale. Municipal insecticide application in the study area is highly variable in space and time, creating a patchy mosaic of selection pressures between blocks. Theory predicts that in such an environment, if the rate of dispersal or migration between areas is greater than the strength of selection, there will be no local adaptation and the allele with the best average fitness across habitats will increase towards fixation, homogenizing populations (33). However, if the strength of selection is higher, then local adaptation can occur, with immigration limiting the probability of fixation of an advantageous allele in a given habitat (33). Population genetic studies have shown that *Ae. aegypti* populations in the Yucatan are panmictic (20, 34), suggesting that migration is high and therefore *kdr* frequencies should be similar within towns. Yet, evidence from town-level data (35) and from our study analyzing fine-scale patterns of *kdr* within a town suggest that local adaptation to pyrethroid insecticides is occurring in local pockets.

Our data also show temporal heterogeneity in *kdr* frequencies throughout the year, suggesting that there is rapid evolution of these markers within the city. Overall, city blocks showed an increase in *kdr* frequencies following the wet season and a subsequent decrease following the dry season, with C1534 showing greater variation in time than I1016. During the wet season, our data show that there was an increase in insecticide

application to control disease, subjecting mosquitoes to higher selection pressure than during the dry season. While our limited data prevented us from statistically quantifying the association between insecticide application and *kdr* frequencies, it is likely that increased application during the wet season in response to the dengue, chikungunya and Zika viruses could be responsible for the increase in *kdr* frequencies by the end of the transmission season. A study in Martinique found that insecticide use shaped genetic patterns: after 50 years of deltamethrin use on the island, the authors found that resistant populations (measured through WHO bioassays and I1016) had lower neutral genetic diversity than susceptible populations (36). Additionally, they found significant population structure despite high gene flow, illustrating that insecticide use could be a factor impacting genetic structure (36).

Furthermore, we noted a decrease in *kdr* frequencies following the dry season, a period of reduced insecticide application. This drop in *kdr* frequency may be indicative of a fitness cost associated with the *kdr* mutations (37, 38), though we cannot determine if the fitness cost is due to the *kdr* mutations themselves, a linked genetic marker that produces a less fit phenotype, or metabolic mechanisms of resistance that we did not measure. Nevertheless, it is encouraging that frequencies of *kdr* can be reduced in the field within relatively few mosquito generations, which may give promise to the eventual restoration of susceptibility if *kdr* is highly associated with the resistance phenotype in this population. While we did not measure phenotypic resistance, a previous study found a significant association between the *kdr* mutations (I1016 and C1534) and resistance to deltamethrin in our study town (11). Nevertheless, future studies that also assess

metabolic mechanisms of resistance in addition to *kdr* and phenotypic resistance can strengthen our understanding of resistance evolution.

The fine-scale spatial and temporal heterogeneity displayed in our data have important implications for monitoring susceptibility in field populations. First, we found that when *kdr* frequencies were assessed at the sector level, the variation in time and space was obscured, displaying a more homogeneous pattern. This suggests that when sampling populations to design resistance monitoring plans, it may not be sufficient to only sample one location within a city and extrapolate results to the entire region. The marked spatial variation found in the *kdr* frequencies at the block level requires a sampling design that selects multiple, random blocks to fully characterize *kdr* in an area.

Secondly, we found seasonal changes in *kdr* frequencies, suggesting that cross-sectional studies, or even longitudinal studies that sample at the same timepoint every year, may not capture evolutionary dynamics. While overall yearly changes in *kdr* may be the most important for public health interventions, it is still important to understand the variation that occurs seasonally as it gives insight into mechanisms underlying *kdr* evolution. Two of the main mechanisms of evolution in this system, selection and migration, are occurring at the fine spatial and temporal scale, and therefore data on *kdr* frequencies must be observed at that scale otherwise the ecological and evolutionary patterns will be obscured, as we have demonstrated here. Ultimately, understanding the processes underlying the patterns that are observed in the field will aid both the control of mosquito populations and counter the negative impacts of the rapid evolution of resistance.

Figures and Tables

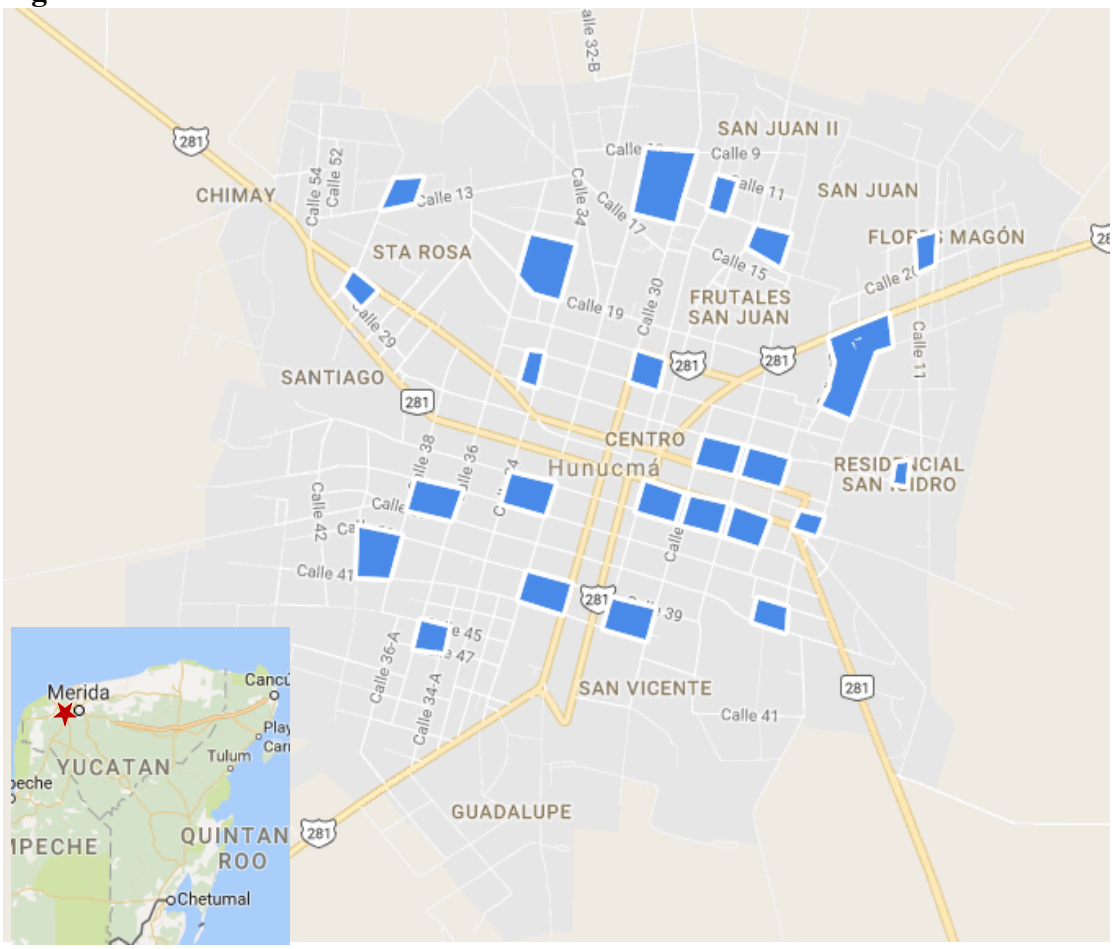

Figure 1.1: Study area. Hunucmá, Yucatán, Mexico. The 24 study blocks are shaded in blue, and the inset shows the location of Hunucma (red star) in the Yucatan Peninsula. (Map data © 2017 Google)

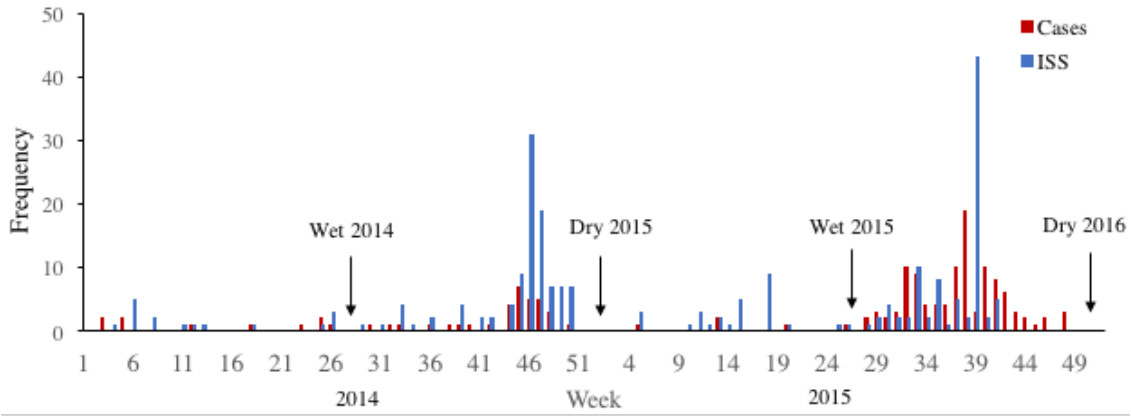

Figure 1.2: Dengue cases in Hunucma and indoor space spraying (ISS) during 2014 and 2015. Sampling points are marked with arrows.

Table 1.1: Summary statistics

Time point	N: females collected	N: males collected	N: total collected	C1534 freq (95% CI)	N: C1534 genotyped	I1016 freq (95%CI)	N: I1016 genotyped
Wet 2014	801	360	1161	0.52 (0.50,0.54)	1070	0.42 (0.40,0.44)	882
Dry 2015	579	354	933	0.67 (0.65,0.69)	764	0.52 (0.49,0.55)	743
Wet 2015	617	334	951	0.58 (0.55,0.61)	708	0.43 (0.40, 0.46)	665
Dry 2016	345	324	669	0.63 (0.60,0.66)	660	0.416 (0.40,0.44)	652
Totals	2,342	1,372	3,714	---	3,202	---	2,942

Figure 1.3: Indoor Space Spraying with deltamethrin. January to June represents the dry season, and the wet season occurs from August – November. Study blocks are marked with an asterisk.

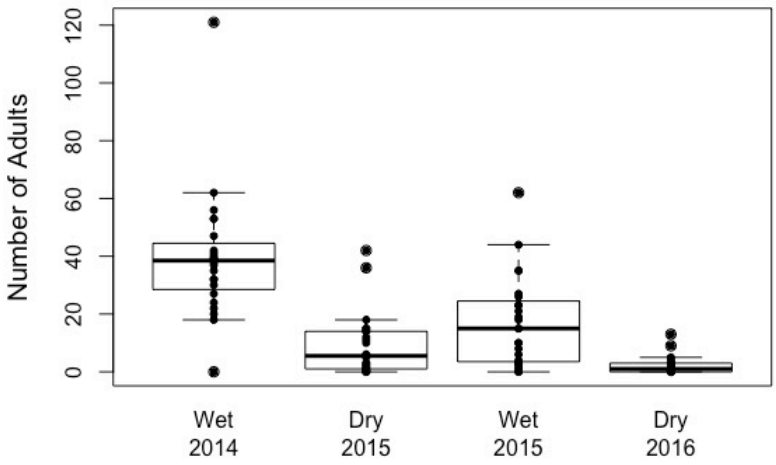

Figure 1.4: Number of adult *Ae. aegypti* caught inside homes during each timepoint. Each point represents a city block.

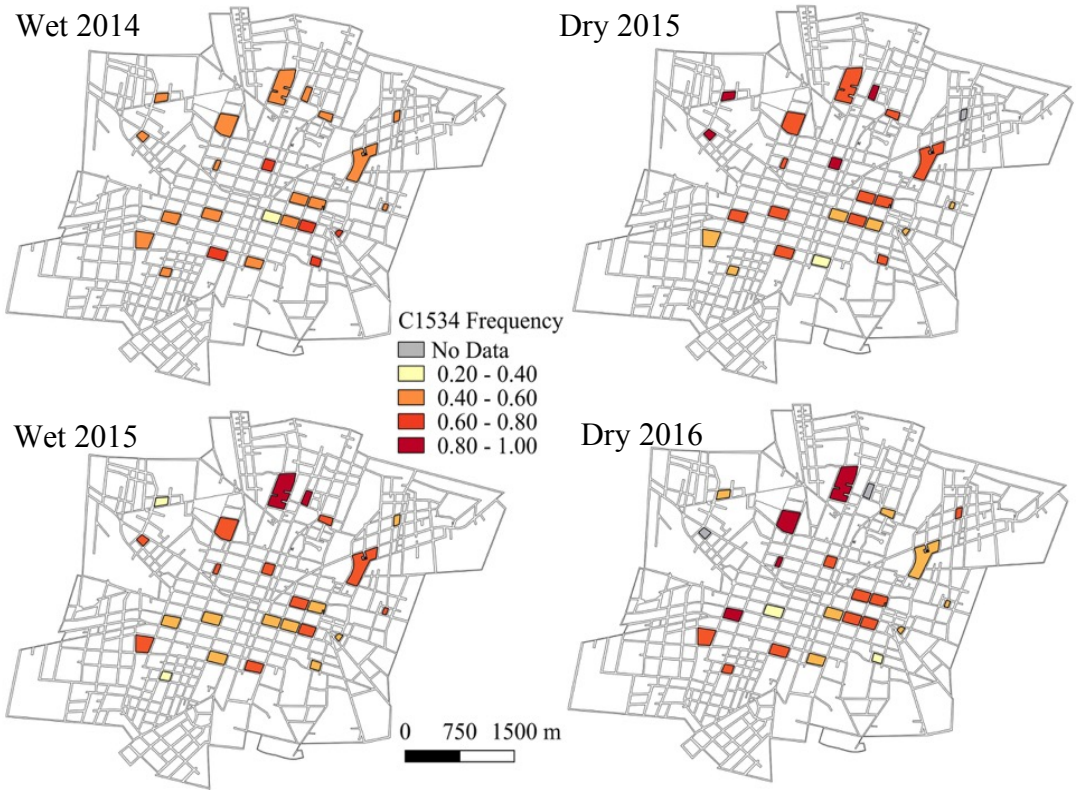

Figure 1.5: C1534 allele frequencies over time. Absence of data at a time-point indicates that no *Ae. aegypti* were collected in that block during that time.

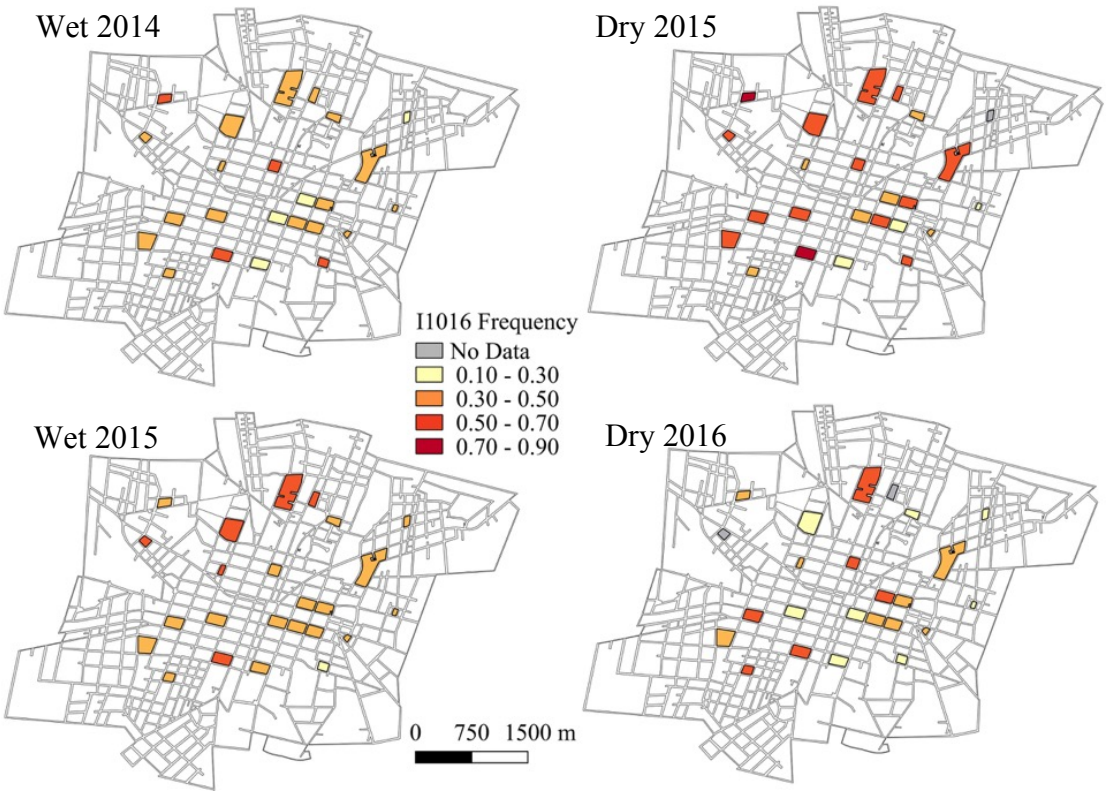

Figure 1.6: I1016 allele frequencies over time. Absence of data at a time-point indicates that no *Ae. aegypti* were collected in that block during that time.

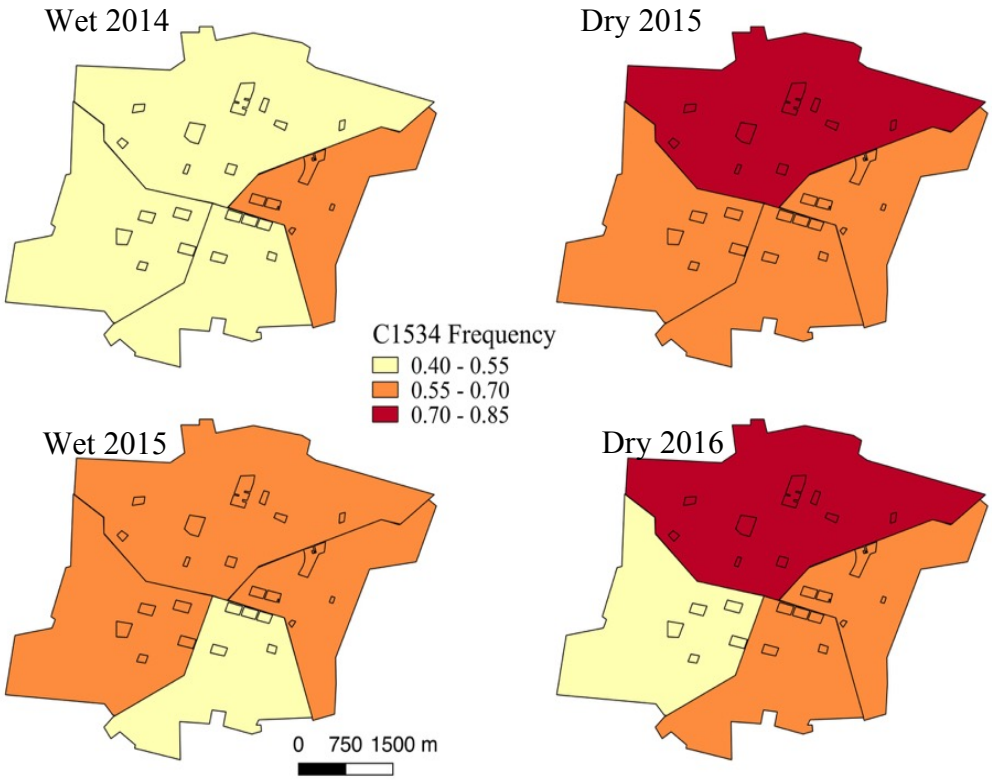

Figure 1.7: C1534 Frequencies over time at the sector level.

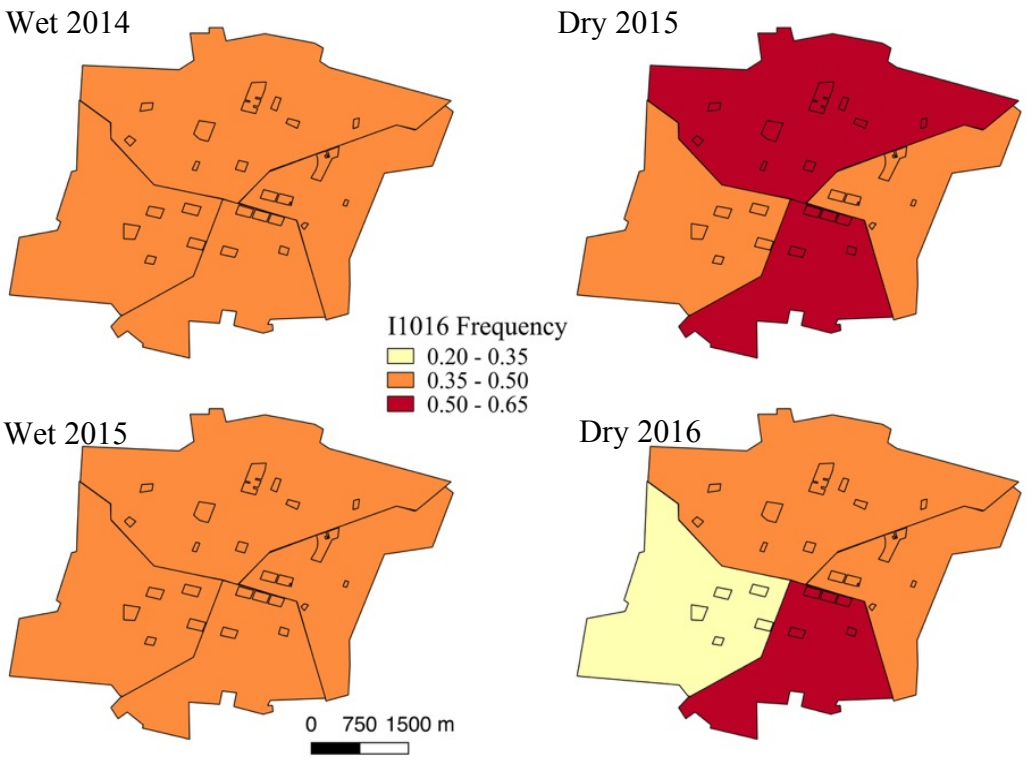

Figure 1.8: I1016 Frequencies over time at the sector level.

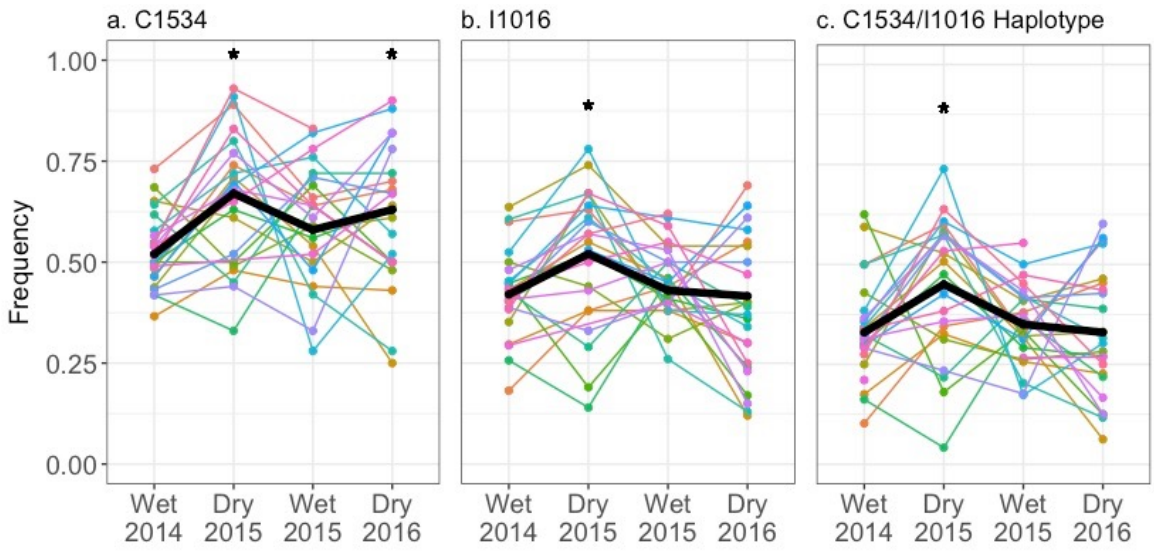

Figure 1.9: *Kdr* mutation and haplotype frequencies over time. Each colored line represents a study block (N=24) and the thick black line represents the mean. A significant difference ($p<0.05$) in allele frequency between a timepoint and the first collection (July 2014) is marked with an asterisk. The absence of data for a block at a timepoint means that no *Ae. aegypti* could be collected from that block during that time.

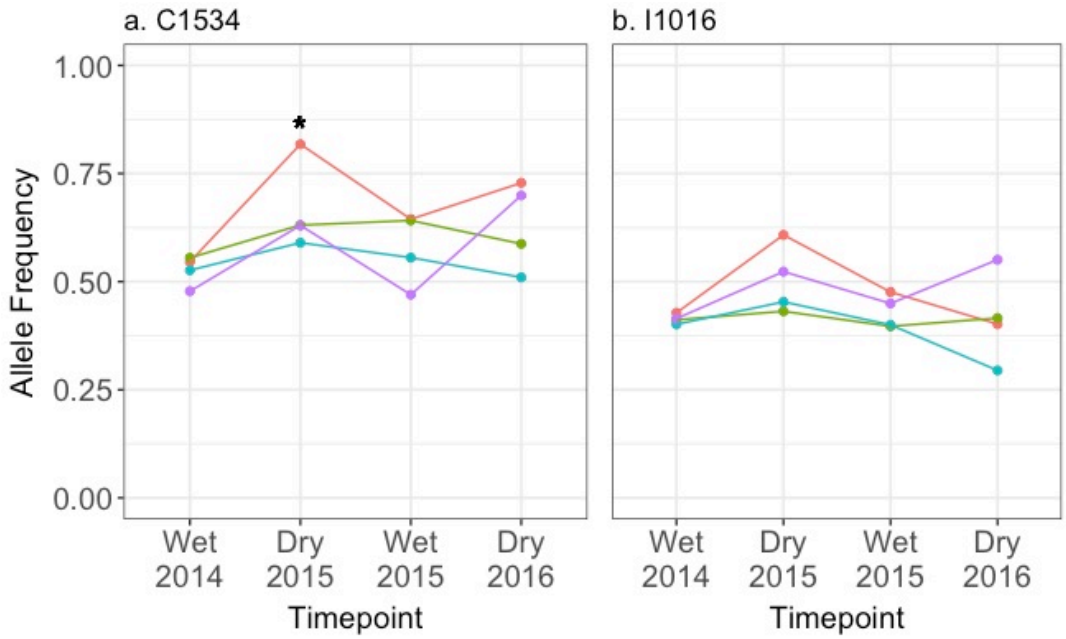

Figure 1.10: The frequency of *kdr* mutations over time at the sector level. The asterisk marks statistical significance ($p<0.05$).

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Supplemental Information

Table S1.1: Allele-specific primer sequences to detect the genotype at 1016 and 1534

Primer Name	**Primer sequence (5' – 3')**
Val1016-f	GCGGGCAGGGCGGCGGGGGCGGGGCCACAAATTGTTTCCCACCCGCACCGG
Iso1016-f	GCGGGCACAAATTGTTTCCCACCCGCACTGA
Iso1016-r	GGATGAACCSAAATTGGACAAAAGC
Phe1534-f	GCGGGCTCTACTTTGTGTTCTTCATCATATT
Cys1534-f	GCGGGCAGGGCGGCGGGGGCGGGGCCTCTACTTTGTGTTCTTCATCATGTG
Cys1534-r	TCTGCTCGTTGAAGTTGTCGAT

Table S1.2: C1534 Frequencies in each block sampled over time. A multiple proportions test following a Chi-square distribution was used to test the difference in frequencies over time for each block and between blocks at each timepoint.

C1534	**Wet 2014**		**Dry 2015**		**Wet 2015**		**Dry 2016**		
Block	**Freq**	**N**	**Freq**	**N**	**Freq**	**N**	**Freq**	**N**	**p-value**
A	0.73	26	0.89	40	0.66	46	0.70	22	0.006*
B	0.48	95	0.74	36	0.64	54	0.68	45	<0.001*
C	0.37	82	0.48	25	0.44	39	0.43	54	0.446
D	0.46	42	0.71	39	0.54	12	0.25	8	0.001*
E	0.65	33	0.61	19	0.50	25	0.64	42	0.333
F	0.44	40	0.68	19	0.58	49	0.61	40	0.034*
G	0.69	54	0.49	40	0.59	43	0.48	40	0.011*
H	0.50	39	0.50	42	0.69	27	0.50	6	0.127
J	0.50	19	0.63	23	0.56	49	0.63	49	0.448
K	0.42	37	0.33	6	0.64	44	0.50	24	0.023*
L	0.62	34	0.45	47	0.72	25	0.72	9	0.005*
M	0.64	35	0.80	44	0.42	33	0.28	16	<0.001*
N	0.58	32	0.72	46	0.76	29	0.57	41	0.038
P	0.53	41	0.91	44	0.28	20	0.52	22	<0.001*
Q	0.50	28	0.69	8	0.82	11	0.88	24	<0.001*
R	0.47	43	0.70	58	0.48	21	0.82	38	<0.001*
S	0.43	37	0.52	51	0.71	12	0.67	39	0.011*

T	0.42	49	0.44	32	0.33	35	0.78	32	<0.001*
U	0.54	52	0.77	28	0.61	18	0.82	20	0.002*
V	0.57	54	0.68	28	0.64	18	0.50	15	0.328
W	0.49	51	--	0	0.52	30	0.67	41	0.038*
X	0.54	56	0.93	30	0.83	15	--	0	<0.001*
Y	0.54	61	0.65	30	0.78	20	0.90	31	<0.001*
Z	0.53	30	0.83	29	0.64	33	--	0	0.001*
***p*-value**	<0.001*		<0.001*		<0.001*		<0.001*		

Table S1.3: I1016 Frequencies in each block sampled over time. A multiple proportions test following a Chi-square distribution was used to test the difference in frequencies over time for each block and between blocks at each timepoint.

I1016	**Wet 2014**		**Dry 2015**		**Wet 2015**		**Dry 2016**		
Block	**Freq**	**N**	**Freq**	**N**	**Freq**	**N**	**Freq**	**N**	**p-value**
A	0.60	25	0.63	43	0.40	46	0.69	21	0.003*
B	0.18	22	0.38	34	0.44	51	0.55	43	<0.001*
C	0.30	27	0.38	24	0.38	37	0.30	54	0.562
D	0.43	22	0.55	37	0.46	12	0.12	8	0.019*
E	0.64	11	0.74	25	0.54	23	0.54	42	0.100
F	0.35	27	0.67	6	0.38	46	0.40	41	0.238
G	0.50	53	0.44	32	0.31	42	0.40	40	0.066
H	0.45	39	0.19	37	0.42	24	0.17	6	0.002*
J	0.45	19	0.52	27	0.41	48	0.36	49	0.270
K	0.26	37	0.14	7	0.46	41	0.24	25	0.006*
L	0.43	34	0.29	52	0.50	25	0.39	9	0.060
M	0.61	33	0.67	47	0.26	33	0.13	15	<0.001*
N	0.45	32	0.61	47	0.45	22	0.34	38	0.007*
P	0.52	41	0.78	47	0.38	13	0.37	19	0.001*
Q	0.45	28	0.64	7	0.61	9	0.58	24	0.349
R	0.40	42	0.53	58	0.45	20	0.64	38	0.019*
S	0.41	37	0.60	31	0.50	12	0.50	39	0.175

T	0.39	49	0.33	32	0.40	34	0.61	32	0.007*
U	0.48	52	0.57	30	0.53	15	0.15	20	<0.001*
V	0.41	54	0.43	30	0.50	17	0.23	15	0.162
W	0.29	51	--	0	0.40	31	0.30	41	0.313
X	0.40	56	0.57	30	0.62	16	--	0	0.027*
Y	0.43	61	0.50	30	0.55	19	0.47	31	0.600
Z	0.38	30	0.67	30	0.59	29	--	0	0.006*
***p*-value**	<0.001*		<0.001*		0.032*		<0.001*		

Table S1.4: *Kdr* frequencies between sectors at each timepoint.

	Wet 2014			Dry 2015			Wet 2015			Dry 2016		
Sec	C1534	I1016	N	C1534	I1016	N	C1534	I1016	N	C1534	I1016	N
1	0.55	0.43	395	0.82	0.61	236	0.64	0.48	187	0.73	0.40	173
2	0.56	0.41	136	0.63	0.43	153	0.64	0.40	184	0.59	0.42	166
3	0.53	0.40	152	0.59	0.45	139	0.56	0.40	180	0.51	0.29	151
4	0.48	0.41	158	0.63	0.52	173	0.47	0.45	99	0.70	0.55	158
p	0.178	0.868		<0.01*	<0.01*		<0.01*	0.089		<0.01*	<0.01*	

Table S1.5: Linear mixed-effects model with C1534 Frequency at the block level as outcome, timepoint as the fixed effect and block as random effect. Reference is wet season of 2014.

Fixed effects	Estimate	St Error	t	p
Intercept	0.523	0.030	17.8	
Dry 2015	0.132	0.040	3.32	0.001*
Wet 2015	0.073	0.039	1.85	0.068
Dry 2016	0.087	0.040	2.18	0.033*
Random effect	Intercept StdDev	Residual StdDev		
Block	0.051	0.136		

Table S1.6: Linear mixed-effects model with I1016 Frequency as outcome with timepoint as the fixed effect and block as random effect. Reference is wet season of 2014.

Fixed effects	Estimate	St Error	t	p
Intercept	0.425	0.028	15.1	
Dry 2015	0.088	0.037	2.37	0.021*
Wet 2015	0.031	0.037	0.84	0.406
Dryt 2016	-0.045	0.037	-1.21	0.231
Random effect	Intercept StdDev	Residual StdDev		

Block	0.054	0.126		

Table S1.7: Linear mixed-effects model with I1016/C1534 haplotype frequency as outcome, timepoint as fixed effect and block as random effect. Reference is wet season of 2014.

Fixed effects	Estimate	St Error	t	p
Intercept	0.337	0.029	11.6	
Dry 2015	0.111	0.040	2.79	0.007*
Wet 2015	0.014	0.040	0.36	0.718
Dry 2016	-0.010	0.040	-0.24	0.809
Random effect	Intercept StdDev	Residual StdDev		
Block	0.037	0.137		

Table S1.8: Linear mixed-effects model with C1534 Frequency at the sector level as outcome, timepoint as the fixed effect, and sector as random effect. Reference is wet season of 2014.

Fixed effects	Estimate	St Error	t	p
Intercept	0.526	0.042	12.4	
Dry 2015	0.141	0.048	3.91	0.017*
Wet 2015	0.052	0.048	1.06	0.316
Dry 2016	0.105	0.048	2.17	0.058
Random effect	Intercept StdDev	Residual StdDev		
Sector	0.050	0.068		

Table S1.9: Linear mixed-effects model with C1534 Frequency at the block level as outcome, season as fixed effect and block as random effect. Reference is the dry season.

Fixed effects	Estimate	St Error	t	p
Intercept	0.636	0.023	27.8	
Wet season	-0.073	0.028	-2.56	0.0130*
Random effect	Intercept StdDev	Residual StdDev		
Block	0.050	0.138		

Table S1.10: Linear mixed-effects model with C1534 Frequency at the sector level as outcome, season as fixed effect and sector as random effect**.** Reference is the dry season.

Fixed effects	Estimate	St Error	t	p
Intercept	0.649	0.034	18.7	
Wet season	-0.097	0.034	-2.88	0.015*

Random effect	Intercept StdDev	Residual StdDev		
Sector	0.050	0.067		

Table S1.11: Linear mixed-effects model with I1016 Frequency as outcome, season as fixed effect and block as random effect. Reference is the dry season.

Fixed effects	Estimate	St Error	t	p
Intercept	0.447	0.022	19.8	
Wet season	-0.006	0.028	-0.22	0.824
Random effect	**Intercept StdDev**	**Residual StdDev**		
Block	0.051	0.136		

Table S1.12: Linear mixed-effects model with I1016 frequency at the sector level as outcome, season as fixed effect and sector as random effect. Reference is the dry season.

Fixed effects	Estimate	St Error	t	p
Intercept	0.460	0.029	15.9	
Wet season	-0.038	0.032	-1.17	0.267
Random effect	**Intercept StdDev**	**Residual StdDev**		
Sector	0.035	0.064		

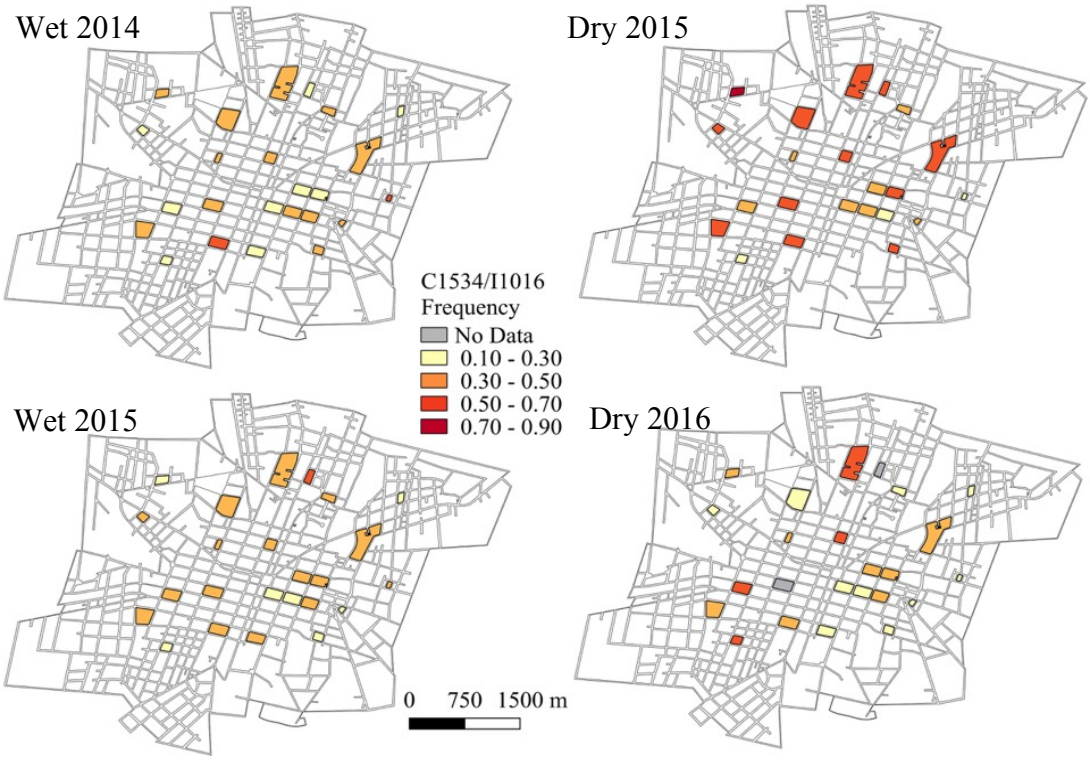

Figure S1.1. C1534/I1016 Haplotype frequencies over time.

Chapter 2: Intraspecific competition mediates evolution of insecticide resistance in *Aedes aegypti*

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Introduction

Insecticide resistance poses a significant threat to the control of both agricultural pests and vectors of human disease. Unfortunately, insecticides remain one of the primary methods for the prevention and control of vector-borne diseases, making it crucial to curtail resistance evolution (1). How can we effectively control vector populations with insecticide while mitigating resistance evolution? Current strategies that are commonly used vary insecticide application in time and/or space (2, 3), with the assumption that a cessation of application will restore susceptibility to that insecticide. This assumption, however, relies on a fitness cost to resistance: if resistant individuals are less fecund or have a longer development time compared to susceptible individuals, susceptible individuals will begin to dominate the population and the insecticide will regain effectiveness. Surprisingly, fitness costs to resistance in *Aedes aegypti*, the main mosquito vector for dengue, chikunguyna, and Zika, are not well understood.

Ae. aegypti experiences strong insecticide selection pressure from vector control efforts that are currently the only way to prevent disease outbreaks. Most *Ae. aegypti* control programs throughout the world employ ultra-low volume spraying (ULV), indoor residual spraying (IRS), and the application of larvicides to target individuals in both the aquatic and terrestrial life stages (4, 5). Consequently, *Ae. aegypti* has developed resistance to every class of insecticide, with the most widespread being pyrethroid

resistance (6). The most common mechanism conferring resistance to pyrethroids is called “knock down resistance,” or *kdr*. These are point mutations in the *para*-orthologous sodium channel gene that alter the ability of the insecticide to bind to the voltage-gated sodium channels in the mosquito’s nerve cell membranes (7). While the frequency of *kdr* mutations are increasing in time and space in many countries (8-11) there continues to be heterogeneity in frequencies across fine spatial scales (12-14) and few populations have reached fixation. How do *Ae. aegypti* populations maintain polymorphism at *kdr* sites, given frequent exposure and strong selection imposed by insecticides? This may be due to a high degree of population mixing paired with heterogeneous selection pressures (13, 15, 16), given a fitness cost to resistance. If so, can we utilize this mechanism to control the evolution of resistance and revert populations to susceptibility?

In this study, we investigated if there is a fitness cost to pyrethroid resistance in *Ae. aegypti* and if intraspecific competition, induced by density-dependence, mediates such fitness cost. It has been well established that density-dependent growth at the larval stage is one of the main factors shaping *Ae. aegypti* population dynamics (17-20). In natural conditions, the *Ae. aegypti* life cycle, which involves four free-living aquatic larval stages and a pupal stage that does not feed, occurs mainly in man-made containers such as buckets or flower pots (21). During the larval period, strong indirect competition for resources occurs, particularly in larval habitats with limited food availability (17, 18, 22). At high larval density, individuals become smaller, develop slower, and have decreased adult survival (17-19). What is unknown is if intraspecific competition affects the performance and fitness of insecticide resistant or susceptible individuals differently,

thereby impacting resistance evolution. For example, if alleles that confer resistance carry a fitness cost, as many do (23), and the fitness cost includes a competitive cost at high population density, then it can lead to the reduction of resistance in the absence of insecticide pressure. Raymond et al. (24) found that under high density conditions, a Cry1Ac (*Bacillus thuringiensis* toxin) resistant population of diamondback moth (*Plutella xylostella*) had reduced survival compared to the susceptible population, and the resistant population also experienced a significant decline in phenotypic resistance in only three generations. A similar competition cost was found in fenitrothion-resistant oriental fruit flies (*Bactrocera dorsalis*): individuals experienced higher survival in the absence of resource competition with susceptible oriental fruit flies (25). Contrary to these findings, Kence and Jdeidi (26) found that heterozygous resistant house flies (*Musca domestica*) had increased survival when in competition with susceptible individuals at high density than without competition in the absence of the insecticide malathion, yet the pattern was reversed in the presence of insecticide. Taken together, these studies suggest that density-dependent competition for resources can modulate fitness parameters.

Using *kdr* as a marker of resistance, we investigate how intraspecific competition at the larval stage can mediate the evolution of resistance in *Ae. aegypti*. Specifically, we aim to test the hypothesis that density-dependent competition can reduce the allelic frequency of *kdr* in a population given a fitness cost to the *kdr* alleles. By using a full-factorial experimental design under semi-natural conditions, we show that density can decrease phenotypic and genotypic resistance in field-derived populations of *Ae. aegypti*.

Methods

To quantify the impact of larval intraspecific competition, specifically between resistant and susceptible individuals, on the resulting adult fitness and insecticide resistance status, we created a full factorial experiment with two factors: density and population. Experiments were conducted in Merida, Mexico during February-May, 2016, inside an urban residence to recreate the typical environmental fluctuations experienced by *Ae. aegypti* populations. Temperature ranged from 21.9ºC to 37.1ºC and humidity from 40% to 82% throughout the course of the experiment.

Ae. aegypti strain description

Pyrethroid susceptible and resistant *Ae. aegypti* field colonies were generated from eggs collected in the cities of Monterrey (Nuevo Leon State, Mexico, susceptible strain) and Uman (Yucatan State, Mexico, resistant strain). Initial gene frequencies for each colony for the C1534 and I1016 *kdr* mutations were quantified using allele-specific real time PCR on 50 randomly selected F1 mosquitoes, applying the protocols described in Alvarez et al. (27). The resulting frequencies of the *kdr* mutations in each population at the beginning of the experiment are listed in Table S2.1, and additional information on the resistant strain can be found in Deming et. al (12). Rates of phenotypic resistance to the pyrethroid permethrin were estimated using the standardized CDC bottle bioassays following published guidelines on 100 F1 mosquitoes of each colony (28).

Using a pyrethroid-susceptible field population of *Ae. aegypti* as opposed to a highly inbred susceptible laboratory strain is a strength of our experiment because neither strain has been selected for laboratory conditions. We also acknowledge, though, that our

experimental strains are derived from different locations and therefore do not share the same genetic background, making it harder to attribute findings to the presence of resistance alleles. However, our main question of interest is whether intraspecific competition can mediate resistance phenotype and genotype, which is an effect that is assessed within a population instead of between populations. Furthermore, density-dependence is a pervasive phenomenon limiting *Ae. aegypti* field populations (17, 19, 22), and therefore we assume that both parental populations were exposed to similar density-dependent pressures in the field.

Experimental Design

There were two levels of density, low (50 larvae) and high (500 larvae), which represent the lower and upper range of larval density described in Merida during the dengue transmission season (12, 29). We used F1 larvae from two *Ae. aegypti* field populations with varying levels of resistance: susceptible (10% frequency of both *kdr* mutations and 100% knock-down to permethrin at the diagnostic time), intermediate (a 50/50 mixture of the susceptible and resistant populations), and resistant (98% frequency of C1534 mutation, 73% of I1016 mutation, and 13% knock-down to permethrin at the diagnostic time). Our experimental design thus involved six treatment combinations (2 densities and 3 resistance levels), which were replicated five times each (Figure S2.1).

We used 2L white experimental buckets, which is the typical size habitat for *Ae. aegypti* in Merida, and filled each one with 1L of municipal water (29). We placed either 50 or 500 first instar larvae from each population into an experimental bucket. Larvae were fed 50mg of bovine liver powder (MP Biomedicals, LLC) every other day until all

reached pupation. Buckets were covered with a mesh net to protect from oviposition of ambient mosquitoes and entrance of other organisms. The number of pupae and recently emerged adults in each bucket were counted daily. Adults were removed daily with a mouth aspirator and placed in an experimental cage (BugDorm-1 Insect Rearing Cage, MegaView Science) and given 75-100ml of 5-10% sugar solution every fourth day for hydration and nourishment.

Once all mosquitoes emerged in the low density treatment, 15 females and 15 males from each replicate were selected at random and transferred into a new mesh-covered bucket containing two oviposition traps and sugar solution for fecundity trials. Oviposition traps were created with a shallow cup (2.5cm depth and 5cm diameter) and lined with coffee filter paper and water to collect eggs. Females were blood-fed twice by a human, with the feedings separated by one day, and left to lay eggs for 3 consecutive days after the last feeding to determine fecundity in the first gonotrophic cycle. On day three, all mosquitoes were euthanized by freezing and stored individually in 100% ethanol until further genotyping analysis. All eggs that were laid were left to desiccate for one day and then placed in municipal water in a 1L bucket with 0.5 teaspoon of liver powder for hatching. We counted larvae that hatched for two subsequent days to determine hatch rate, and then calculated the number of viable offspring (fertility), defined as fecundity multiplied by the hatch rate. For the high density treatment, the same procedures were conducted, however half of the 30 mosquitoes (7 males and 7 females) were removed halfway through the experiment (day 18) for fecundity trials because the total time for individuals to emerge as adults was between 30-40 days. Obtaining two

samples over the duration of the high density experiment minimized any potential bias if emergence time differed between susceptible and resistant individuals.

Phenotypic resistance assay

CDC bottle bioassays were conducted on mosquitoes from each treatment replicate to determine phenotypic resistance. Four replicates of 25 mosquitoes each were placed in bottles coated with 15mg/ml of technical grade permethrin according to CDC guidelines (28). In the high density treatment, two of the four replicates were conducted at day 18, using mosquitoes that had emerged prior to that date, as to not bias results as previously stated; the other two replicates were completed at the end of the experiment, which was between days 33-40 depending on the replicate. The low density treatment did not contain enough mosquitoes for the bottle bioassays since they only contained 50 mosquitoes at maximum and 30 were sampled for fecundity trials, so two extra replicate buckets were simultaneously run but only used to complete the bioassays. The number of individuals knocked-down were recorded every 10 minutes for 120 minutes or until all individuals were knocked-down. The percentage of individuals knocked down at the diagnostic time of 30 minutes was calculated for each replicate, and phenotypic resistance was defined as this percentage.

Genotype analysis

We extracted the DNA from each of the 30 individuals per replicate that were sampled for fecundity trails using the salt extraction method (30), and we conducted

allele-specific real time PCR to determine genotype at the 1534 and 1016 locus following protocols described in Alvarez et al. (27).

Statistical Analyses

Allele frequencies for C1534 and I1016 were calculated for each population before and after the experiment. To test for linkage disequilibrium between the two markers, we calculated the coefficient D and the resulting r^2 following the equations outlined in Gillespie (31) and used a Chi-square test with one degree of freedom to test the statistical significance. Fecundity was determined by calculating the average number of eggs laid per female per replicate, and fertility was determined by multiplying fecundity by the proportion of eggs that hatched. We analyzed the effect of resistance level, density, and their interaction on each life history parameter. For development time, defined as the total number of days from first instar larva to adult, we used a linear mixed effects model with replicate as a random intercept (R package nlme(32)). The probability of survival was analyzed using a generalized linear mixed effects model with binomial errors (GLMM), also with replicate as a random intercept (R package lme4 (33)). Fecundity was calculated per replicate (not per individual), and because it was Poisson-distributed with over-dispersion, we used a negative binomial GLM (R package MASS (34)). To quantify the relationship between fecundity and allelic frequency, we used a simple linear regression for each mutation separately. The change in genotype was analyzed with a chi-square test of independence, and the difference between densities in the proportion knocked-down with insecticide was assessed with a Welch t-test. Analyses were conducted with the R statistical program(35).

Results

Larval performance

The mean (±standard deviation) number of days from first instar larva to adult was 12.3±0.6 days longer at high density than low density (GLMM generalized linear mixed-effects model, $t=21.93$, $p<0.0001$), with a statistically significant interaction occurring between density and resistance level ($t=5.13$, $p<0.0001$) (Figure 2.1a, Table S2.2). At low density, there was no difference between resistance levels in time to adult emergence, with a mean development time for all populations of 6.9± 0.25 days (Table 1). However at high density, the resistant population took on average 4.0±0.8 days longer to develop than the susceptible and intermediate populations (Table 2.1). The survival probability from first instar larva to adult was significantly lower for individuals in the high density treatment than the low density (GLMM, odds ratio = 0.15, 95%CI = 0.11, 0.20, Table S2.3) and significantly higher for the resistant population compared to the susceptible across both density treatments (OR = 2.3, 95% CI = 2.0, 2.6) (Figure 2.1b). Individuals in the intermediate population had an increased probability of survival at low density compared to the susceptible population (GLMM, OR = 11.0, 95%CI = 4.32, 37.18), yet a decreased survival probability at high density (OR = 0.70, 95%CI = 0.62, 0.78) (Figure 2.1b).

Adult fitness

The resistant population laid significantly fewer eggs and fewer viable eggs than the susceptible population during the first gonotrophic cycle at both densities (Figure 2.2). At low density, the resistant population laid 7.3 ±5.1 eggs compared to and 32.2

±14.5 in the susceptible population, representing a 78% decrease in fecundity. At high density, there was an even greater reduction: the resistant population only laid 1.8 ±1.2 eggs per female compared to 20.7 ±10.7 in the susceptible population, which is a 92% decrease in fecundity for the first gonotrophic cycle. The intermediate population, however, suffered a 76% decrease in fecundity between densities, dropping from 30.9±8.4 eggs at low density to 7.5 ±5.7 eggs at high density. Overall, there was a significant effect of resistance level on the mean number of eggs laid per female (GLMM, resistance level $z= -6.71$, $p<0.0001$), and a significant negative interaction between density and resistance (GLMM, $z= -3.22$, $p=0.0013$, Table S2.4). Controlling for density, the resistant population laid 4.3±0.08 less eggs than the susceptible population in the first gonotrophic cycle.

The hatch rate of eggs varied greatly between experimental replicates within the same density treatment and population, therefore causing fertility to also vary greatly (Figure 2.2b). However, it is important to note that the resistant population only produced 0.74±0.43 viable offspring at high density compared to 3.1±4.1 in the intermediate population and 12.9±10.7 in the susceptible population, suggesting negative population growth over time for the resistant population.

Regression analyses revealed a negative association between fecundity and the frequency of both C1534 and I1016 mutations (Figure 2.3). Fecundity was reduced by 2.7±1.9 eggs per female for each 0.1 increase in allele frequency of C1534 at high density ($F_{1,13}=9.2$, $p=.0095$, $R^2=0.37$) and a reduction of 3.0±1.5 eggs at low density (Figure 2.3; $F_{1,13}=19.4$, $p=.0007$, $R^2=0.57$). Similarly, there was an estimated reduction of 2.3 ±1.5 eggs per female for each 0.1 increase in allele frequency of I1016 at high density

($F_{1,13}$=10.3, p=.0067, R^2=0.40) and 4.8 ±1.9 eggs at low density (Figure 2.3; $F_{1,13}$=31.4, p=8.64e-05, R^2=0.68).

Adult genotypic and phenotypic resistance

Phenotypic resistance decreased significantly in the resistant population at high density compared to low (Figure 2.4, Welch t-test, t= -3.41, df = 4.5, p-value = 0.0225). At low density, only 48.2% (±28.5%) of the resistant population was knocked-down at 30 minutes, but at high density, 93% (±7.1%) were knocked-down. This reduction in phenotypic resistance at high density rendered the "resistant" population susceptible according to the WHO guidelines, which mark the resistance threshold at 80% population knock-down at the diagnostic time (Figure S2.2 (28)).

At both densities, the C1534 allele frequency of the resistant population was significantly reduced from a starting frequency of 0.98 to 0.93±0.05 at low density (Fisher's exact test, p=0.003) and to 0.69±0.04 at high density (Fisher's exact test, p<0.0001) (Figure 2.5). This marked effect of density on phenotypic frequency was not observed for the I1016 mutation (Figure 2.5). The frequency of the I1016 allele increased slightly after both treatments though not significantly (low density: Chi-square = 0.47, p=0.492; high density: Chi-square = 3.3, p=0.068). Allele frequencies for C1534 and I1016 were analyzed separately since we found that they were not in linkage disequilibrium (D=0.02, r^2= 0.0072, Chi-square=0.943, p=0.331).

Discussion

We found that intraspecific competition can act as a selective force to regain susceptibility in pyrethroid-resistant *Ae. aegypti* mosquitoes. High density larval conditions induced competition, evidenced through reduced immature survival, delayed development time, and lower fecundity for all populations. Consequently, this heightened competition selected for individuals without the C1534 *kdr* mutation, causing a striking decrease in its frequency in the resistant population. The frequency of the C1534 decreased almost 30% in only one generation of selection. Such rapid evolution gives insights into the maintenance of polymorphism at *kdr* sites in *Ae. aegypti* field populations. Although insecticide selection pressures are strong, they are rarely uniform in time or space, as they are largely driven by disease outbreaks (36). In the absence of insecticide, population densities may increase, imposing stronger competition and selection towards susceptibility. The alternation of insecticide selection pressure with selection due to density-dependence may, in part, account for the genetic variation at *kdr* loci and can be leveraged to mitigate resistance evolution.

Equally important is that phenotypic susceptibility was re-established in the resistant population through a gene-environment interaction. Larvae from the same parent population with high resistance exhibited different resistance phenotypes depending on the conditions in which they were raised. If raised with minimum intraspecific competition (low density), they remained resistant, however if they were raised under strong competition (high density), they became diagnostically susceptible according to WHO guidelines (28). Not only do these results demonstrate that phenotypes can be altered based on environmental conditions, but they also raise concerns about the external

validity of biological assays used to phenotypically characterize the levels of resistance of natural populations. Based on our findings, we hypothesize that the CDC bottle bioassay and WHO susceptibility test would provide different results if performed with adults collected from the field (that are naturally constrained by food and density) versus adults reared in optimal laboratory conditions. Further evaluations of the bioassay methodology are needed, including the investigation of the correlation between phenotype and genotype at higher insecticide doses and the interplay between density and phenotypic resistance.

Overall, our results suggest a substantial fitness cost to pyrethroid resistance: the resistant population had longer development time and lower fecundity than the susceptible population. While we found a strong negative association between the population *kdr* frequency and fecundity, suggesting that the fitness cost may be due to the *kdr* mutations, we cannot rule out metabolic mechanisms that are also responsible for the resistance phenotype in *Ae. aegypti* (37). However, it has been shown through quantitative trait loci mapping that mutations in the *para* gene (including C1534 and I1016) are largely responsible for knock-down resistance to permethrin and the genes involved in metabolic resistance are more responsible for recovery following insecticide exposure (38). Our measure of phenotype was knock-down time, suggesting that the *kdr* mutations may have played a larger role. Additionally, Saavedra-Rodriguez et. al (39) found that after five generations of permethrin selection in six different strains of *Ae. aegypti*, including five strains from the Yucatan, there was an inverse relationship between the frequency of I1016 and the number of metabolic detoxification genes that were differentially transcribed. In particular, they found that the Merida strain had a high

frequency of I1016 and only had four detoxification genes upregulated. Our resistant strain originated from a satellite town of Merida, leading us to believe that since the frequency of I1016 was high in our population, there would likely be low metabolic resistance as well.

Only two other studies have directly examined fitness costs in *Ae. aegypti* associated with pyrethroid resistance. Brito et al. (40) compared an *Ae. aegypti* strain with the *kdr* mutations on a susceptible genetic background of the Rockefeller laboratory strain to the Rockefeller strain in order to isolate the effect of the *kdr* mutations on fitness parameters. They also found longer development time and lower fecundity that strongly implicate a fitness cost to *kdr* mutations themselves. Martins et al. (41) compared fitness of three field populations selected with deltamethrin for nine generations to another three field populations left unselected, with the Rockefeller susceptible strain as their control. While they also found longer development time and reduced fecundity in the laboratory selected mosquitoes, only one unselected field population exhibited a fitness cost compared to Rockefeller. These results highlight potential differences in laboratory selected mosquitoes compared to field collected mosquitoes, suggesting that it may be prudent to use field collected mosquitoes to assess fitness. Given we used field-derived populations in our study (F1) and performed all our experiments under natural temperature and humidity regimes, we consider our estimates of fitness cost to pyrethroid resistance to be more realistic than previous quantifications.

Although we found longer development times in the resistant population compared to the susceptible, we unexpectedly found greater immature survival in the resistant population. In natural populations, this higher survival may compensate for a

longer development time, which may subject individuals to a higher probability of predation or unfavorable environmental changes. Additionally, the intermediate resistant population, which represented competition between resistant and susceptible individuals, suffered a greater reduction in survival and fecundity in the high density condition than the susceptible or resistant population. Individuals in the high density condition for the intermediate population faced two types of competition: intra- and inter-strain competition. Resistant individuals had to compete with both other resistant individuals and other susceptible individuals, potentially increasing the strength of competition compared to a population of just resistant individuals. On the other hand, susceptible individuals in the intermediate population may have experienced a decrease in overall strength of competition. However, the large decrease in survival and fecundity suggest that all individuals suffered from increased competition.

The strong fitness cost to pyrethroid resistance quantified in our study, combined with the potential for density dependence to slow the resistance evolution provide important insights for the development of resistance management strategies aimed at restoring pyrethroid susceptibility. Relying on the knowledge of resistance management in agroecosystems, we hypothesize that insecticide application strategies that are both heterogeneous in space and time (e.g. the mosaic strategy (2) or the refuge strategy (42)) may be able to exploit the competitive disadvantage and high fitness costs of pyrethroid-resistant mosquitoes. Both of these strategies leave some areas untreated, which can increase density in those areas, increase intraspecific competition, and exploit the fitness costs of resistance in the absence of insecticide. Furthermore, the strong negative impacts of density on *Ae. aegypti* life history traits can reduce overall population abundance, not

just of the resistant population. Further research can also focus on the potential for strategies that affect larval density (e.g. source reduction) as an approach to manage insecticide resistance within an integrated vector management plan. The insightful results of this study can lead to novel control strategies that leverage the effect of density and the fitness costs of resistance in field populations.

Figures and Tables

Table 2.1: Summary of life history parameters for each combination of resistance level and density (mean ± std deviation).

Resistance level	Density	Development time (days)	Survival	N*	Fecundity	Fertility	†Proportion Knocked down	C1534 Frequency	I1016 Frequency
Susceptible	Low	6.9 ±0.08	81±1.1%	135	32.2 ±14.5	16.1±12.6	1.0±0.0	0.06±0.05	0.15±0.07
Intermediate	Low	7.1±0.3	98.4±2.2%	135	30.9 ±8.4	22.0±7.3	0.80±0.17	0.51±0.13	0.49±0.04
Resistant	Low	6.9±0.3	96.0±2.4%	131	7.3 ±5.1	4.5±4.1	0.48 ±0.29	0.93±0.05	0.76±0.05
Susceptible	High	19.2 ± 1.8	66.7±6.1%	157	20.7 ±10.7	12.9±10.7	1.0±0.0	0.07±0.03	0.12±0.07
Intermediate	High	19.7 ± 2.8	58.4±19.4%	160	7.5 ±5.7	3.1 ±4.1	0.98±0.02	0.37±0.16	0.51±0.15
Resistant	High	23.4 ± 1.7	81.3±7.0%	150	1.8 ±1.2	0.74±0.43	0.93 ±0.07	0.69±0.04	0.81±0.02

*Sample size (N) is for the number of individuals used for fecundity trials and then subsequently genotyped.

†The proportion knocked-down refers to the phenotype measured by the CDC bottle bioassay

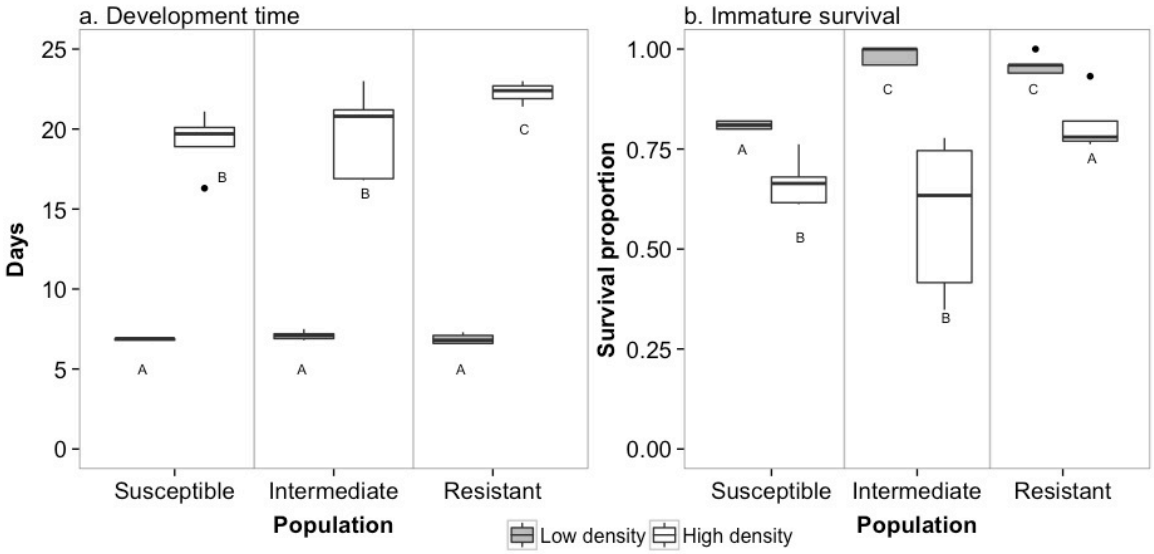

Figure 2.1: Larval performance for each population and density. Outcomes denoted by different letters are statistically different ($\alpha<0.05$) from one another. Boxplots show the distribution of (a) development time, defined as total time from first instar to adult, and (b) immature survival, measured as the proportion of the population surviving to adult, of all five replicates.

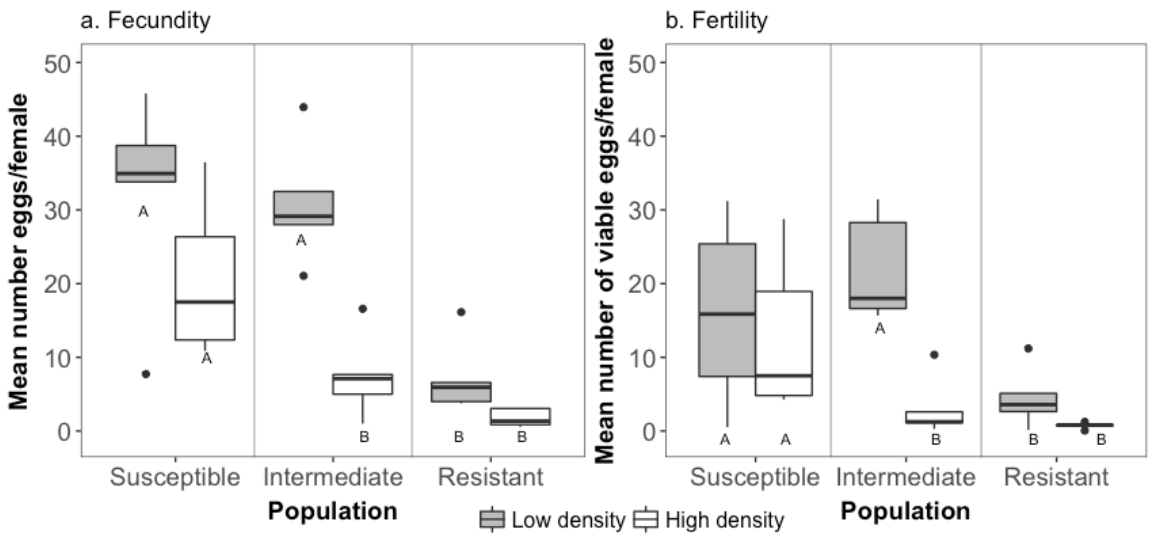

Figure 2.2: Adult fitness for each population and density**.** Outcomes denoted by different letters are statistically different ($\alpha<0.05$) from one another**.** Fecundity and fertility for each replicate were defined as a population measure (the mean number of eggs or viable eggs per female in the first gonotrophic cycle), and boxplots show the distribution of the means for all five replicates.

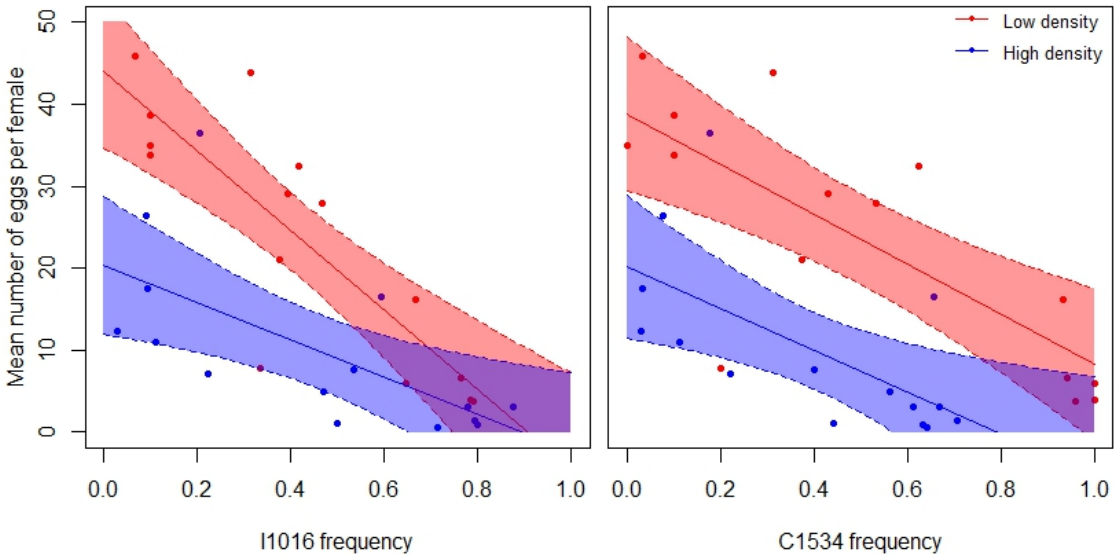

Figure 2.3: Population *kdr* frequency is correlated with the population fecundity. Density conditions are stratified, with low density in red and high density in blue. Each point represents a population. Lines represent the linear regression of the *kdr* mutation on fecundity, and the shaded areas bounded by the dashed lines represent the confidence bounds of the regression.

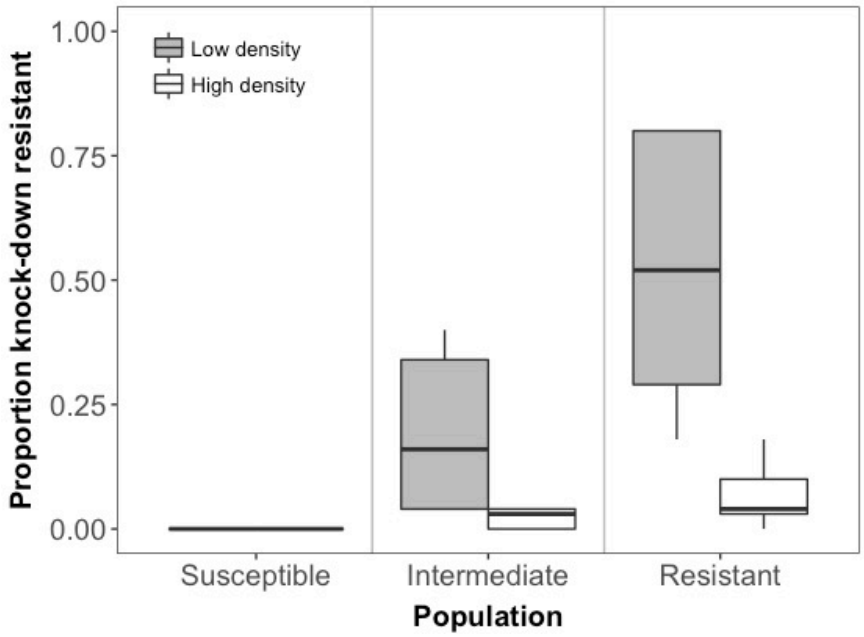

Figure 2.4: Phenotypic resistance changes based on density conditions. Boxplots show the distribution of the proportion of the population knocked-down in the presence of the diagnostic dose of permethrin according to CDC bottle bioassay standard procedures.

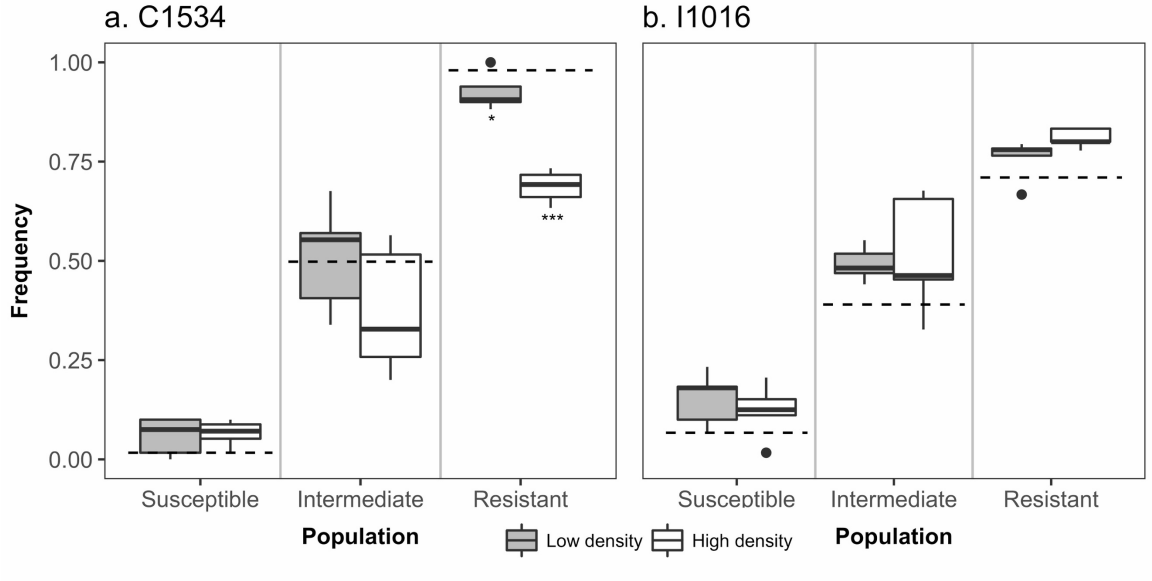

Figure 2.5: *Kdr* frequencies as a result of density-dependent selection. Box plots show the distribution of the *kdr* frequencies of adult mosquitoes emerging from each treatment combination. The dotted line represents the initial frequency of first instar larvae in the population. One asterisk indicates statistical significance at $p<0.01$ and three indicate it at $p<0.0001$.

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Supplementary information

Table S2.1: Initial population descriptions.

Population	Source Location	N	C1534	I1016	% Knock-down to permethrin at 30 min
Susceptible	Cienaga de Flores, Monterrey, Nuevo Leon	51	0.01	0.01	100%
Intermediate	50/50 mix of susceptible and resistant strains	--	0.50	0.36	--
Resistant	San Lorenzo and Acim, Uman, Yucatan	61	0.98	0.71	13.3%

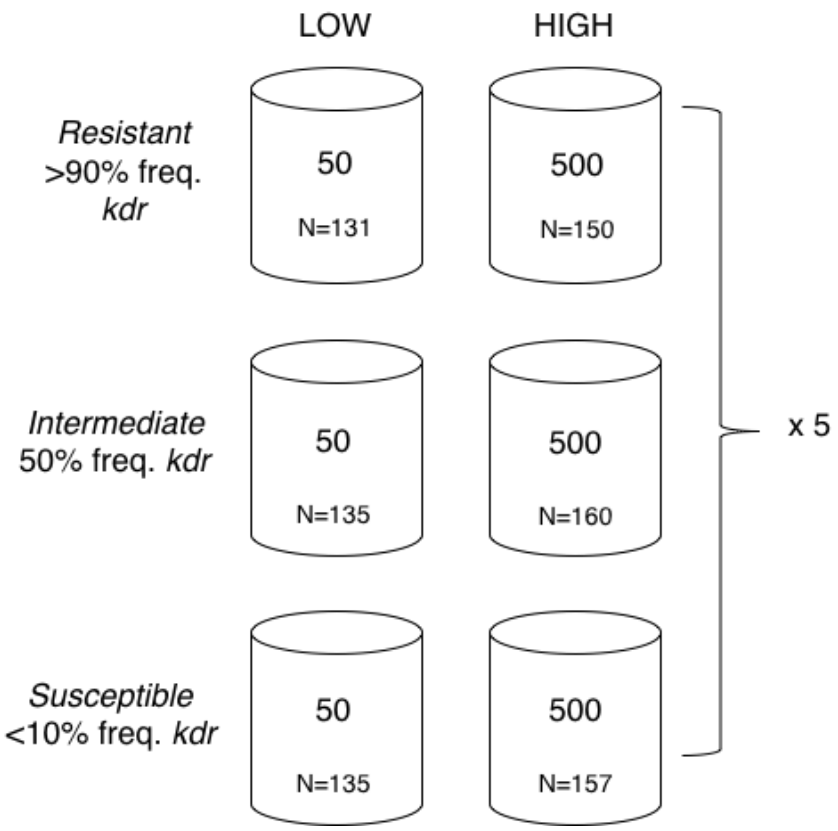

Figure S2.1: Study design. First instar larvae from three populations with varying resistance levels were placed into the following density treatments. The intermediate resistance population was a 50/50 mix of the resistant and the susceptible. All six combinations were replicated five times each. Total number of mosquitoes genotyped is indicated for all 5 replicates combined, though allele frequencies were calculated for each replicate separately.

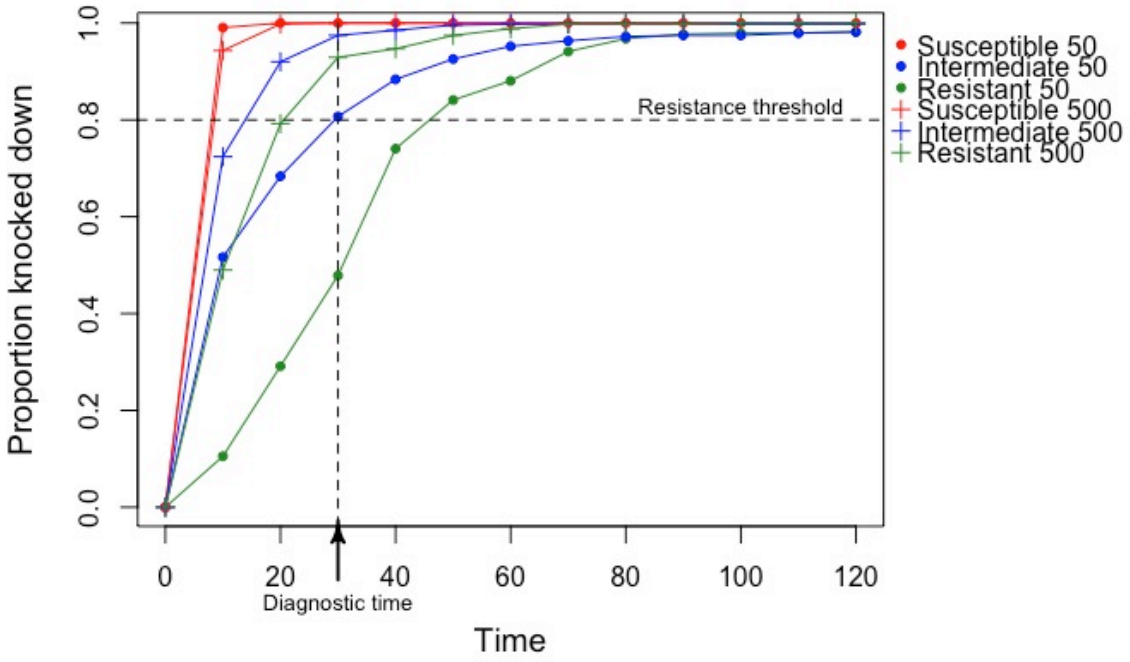

Figure S2.2: Phenotypic resistance changes based on density. The proportion of the population knocked-down at each time timepoint during the 120-minute assay is shown. The diagnostic time is 30 minutes and the resistance threshold according to WHO is 80% (below 80% is resistant; above is susceptible). Red lines are the susceptible population, blue is intermediate, and green is resistant. Circles are low density and crosses are high density.

Table S2.2: Development time as a function of resistance level, density, and their interaction using a linear mixed effects model.

Fixed effects	Estimate	St Error	t	p
Intercept	6.86	0.61	11.21	< 2e-16***
High density	12.35	0.56	21.93	< 2e-16 ***
Intermediate	0.26	0.73	0.356	0.721
Resistant	0.21	0.73	0.283	0.777
High density*Intermediate	0.59	0.78	0.752	0.452
High density *Resistance	3.98	0.78	5.129	< 2e-16 ***
Random effect	Var	St dev		
Replicate	0.683	7.81		

Reference is low density and susceptible population.

Table S2.3: Probability of survival as a function of resistance level and density using a mixed effects model with a binomial distribution.

Fixed effects	Estimate	St Error	Z	p
Intercept	2.56	0.20	12.81	< 2e-16***
High density	-1.89	0.15	-12.81	< 2e-16 ***
Intermediate	-0.29	0.06	-5.00	5.9e-07 ***
Resistant	0.82	0.07	12.38	< 2e-16 ***
Random effect	Var	St dev		
Replicate	0.089	0.300		

Reference is low density and susceptible population.

Table S2.4: Fecundity as a function of resistance level, density, and their interaction using a generalized linear model with a negative binomial distribution.

	Estimate	St Error	Z	p
Intercept	6.18	0.15	40.52	< 2e-16***
High density	-0.32	0.21	-1.50	0.1330
Intermediate	-0.06	0.22	-0.29	0.7710
Resistant	-1.46	0.22	-6.71	1.9e-11***
High * Intermediate	-0.95	0.30	-3.14	0.0017 **
High * Resistant	-0.99	0.31	-3.22	0.0013 **

Reference is low density and susceptible population

Chapter 3: Selection dynamics of the knock-down resistant mutations in *Aedes aegypti*

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Introduction

The widespread prevalence of insecticide resistance in *Aedes aegypti* mosquito populations is an increasing challenge for the control of dengue, Zika, and chikunguyna. Of particular concern is resistance to pyrethroid insecticides, as they are of low mammalian toxicity and currently the only insecticide class approved for use on insecticide-treated materials, such as curtains and screens used for *Ae. aegypti* control (1). Throughout the world, populations are exhibiting high levels of pyrethroid resistance, usually measured by the frequencies of the knock-down resistant (*kdr)* mutations (2-5). These are point mutations in the *para*-orthologous sodium channel gene that disrupt insecticide binding to the voltage-gated sodium channels that usually result in paralysis and death (6). Many *kd*r mutations have been identified, though only two are have been strongly associated with type I pyrethroid resistance in *Ae. aegypti*, the phenylalanine to cysteine mutation in 1534 (F1534C) and the valine to isoleucine mutation in 1016 (V1016I) (4, 7, 8).

The emergence and persistence of pyrethroid resistance has sparked a large body of literature aimed at understanding the genetic mechanisms underlying resistance (9-11), yet the feasibility of restoring susceptibility is largely unknown. Furthermore, despite strong selection pressures from insecticide application that should push the *kdr* mutations towards fixation, polymorphism is maintained at the *kdr* locus in field populations (12-

15). For example, in Chapter 1, we showed that the frequency of *kdr* mutations in populations of *Ae. aegypti* from a satellite city of Merida in the Yucatan, Mexico, were highly heterogeneous in both time and space, and decreased during the dry season when insecticide application was minimal. This suggests that perhaps there is a fitness cost to resistance and that pyrethroid susceptibility can be regained in the absence of insecticide.

While fitness costs in resistant strains of *Ae. aegypti* have been demonstrated (16-18), studies have mostly focused on individual effects, such as developmental traits and reproduction, and very few studies have quantified population effects, such as a restoration of susceptibility with the cessation of insecticide application. Instead, most studies focus on selection towards resistance, using deltamethrin or permethrin to select for *kdr* mutations. For example, in a pair of studies, Alvarez et al. (18, 19) used deltamethrin to artificially select for resistance in *Ae. aegypti* for 15 generations by exposing them to the insecticide for one hour and then rearing the survivors. They found that the I1016 allele increased from 0.02 to 0.5, that the C1534 increased to fixation, and that the resistant mosquitoes had a lower hatch rate and shorter lifespan compared to the unselected, susceptible mosquitoes. Martins et al (16) followed a similar selection protocol and found that after nine generations of deltamethrin selection, the mosquitos had lower larval viability and longer development time, suggesting a cost to resistance. These studies give insight into potential fitness costs associated resistance, though they do not explore how the fitness costs might operate at the population level when insecticide pressure is absent. Here, we ask: given a fitness cost to the C1534 and I1016 *kdr* mutations, can susceptibility be restored in the absence of insecticide?

Methods

Experimental Design

We reared the same population of field-derived *Ae. aegypti* in BugDorm-2120F Insect Rearing Tents (MegaView Science) under two treatments: with insecticide and without insecticide. To incorporate insecticide into the tent, we covered the left and right sides, approximately 3,600cm^2 each, with Pramex™ Long Lasting Insecticidal Nets (MGK) containing Olyset™ Technology with 2% permethrin. We replicated each treatment 5 times, and all replicates were conducted inside a house in Merida, Mexico without temperature or humidity control to simulate semi-natural conditions.

For each replicate, we placed 500-800 eggs into a 2L white bucket containing 1L of municipal water and placed one bucket into each experimental tent. We fed larvae bovine liver powder (MP Biomedicals, LLC) *ad libitum* to ensure sufficient food and minimize the effects of larval competition. Sugar water was provided daily for all emerged adult mosquitoes. Once all mosquitoes emerged, females were blood-fed once a week with human blood for two weeks and allowed to lay eggs into a black oviposition trap, approximately 10cm tall and 5cm in diameter. After 2 weeks, eggs were removed, left to dry for 1 day, and then 500-800 were selected at random and placed into a clean 2L white bucket to hatch and start the next generation. All adult mosquitoes were also removed from the tent after 2 weeks using a Prokopack aspirator (20) and placed in a -20ºC freezer for euthanization. Thirty individuals (15 females and 15 males) were selected at random for *kdr* genotyping. This process was repeated for 10 generations, which was about a year in length. After generation 10, eggs from the tents with

insecticide were placed in the tents without insecticide, and vice versa. The same protocol was then repeated for 5 additional generations.

Phenotypic resistance assays

At generation F0 and F10, we conducted the CDC bottle bioassay using the standard protocol (21) to test for phenotypic resistance to technical grade permethrin at a concentration of 15µg/ml. The proportion of mosquitoes knocked-down at the diagnostic time of 30 min was recorded, and a Welch two-sample t-test was used to determine the difference in knock-down percentages between the treatments.

Molecular assays

Allele-specific real-time PCR determined the *kdr* genotypes at codons 1016 and 1534 for generations 1, 3, 7, and 10. DNA was extracted from individual mosquitoes using a 50ul solution containing 5ul of Taq 10X buffer (containing 500mM KCl, 100mM tris HCl, 15mM MgCl2, and 1% Triton X-100) and 45ul of sterile ddH_2O and heating in an Eppendorf Mastercycler© pro thermocycler at 95ºC for 15 minutes. Allele-specific real time PCR was conducted using a Biorad© CFX96 machine (Hercules, CA) following the protocols outlined in Saavedra et al (8) for 1016 and Yanola et al (22) for 1534.

Strain characterization

Ae. aegypti eggs used for the experiment were generation F1 from eggs originally collected from Itzincab, a satellite city of Merida, Mexico. This population has high

genotypic and phenotypic pyrethroid resistance, making them ideal to test the loss of resistance in the absence of insecticide pressure. The frequencies of I1016 and C1534 in the population were 0.595 and 0.937 respectively, and the population exhibited a 13.7% knock-down rate to permethrin at the diagnostic time according to CDC bioassay protocols.

Analysis

We calculated allele frequencies at the 1534 and 1016 *kdr* loci for each replicate at generations F1, F3, F7, and F10. We assigned 1534 locus A, defining p_{A1} as the frequency of F1534 (the susceptible allele), and 1016 was assigned locus B, with p_{B1} as the frequency of V1016 (the susceptible allele). To look for signatures of selection, we conducted a test of Hardy-Weinberg Equilibrium (HWE) for each locus at each generation by calculating the inbreeding coefficient, F (using 1534 as an example):

$$F = 1 - \frac{obs(A_1A_2)}{2p_{A1}p_{A2}}$$

When F=0, genotype frequencies are as expected under HWE; if $0<F\leq1$, there is an excess of homozygotes, and when $F<0$, there is an excess of heterozygotes (23). The quantity nF^2 follows a Chi-square distribution with one degree of freedom, where n is the number of individuals.

Because these loci are physically close on the chromosome, we calculated linkage disequilibrium between them at each generation and for each replicate. The maximum likelihood estimate of linkage disequilibrium, D, is:

$$D = \frac{1}{n} n_{A1B1} - 2p_{A1}p_{B1} \qquad (24)$$

where *n* is the number of individuals and the digenic count, n_{A1B1} , of $A_1B_1 + A_1|B_1$ is the following sum of genotype counts:

$$n_{A1B1} = 2(A_1A_1B_1B_1) + A_1A_1B_1B_2 + A_1A_2B_1B_1 + \frac{1}{2}(A_1A_2B_1B_2) \qquad (24)$$

Using *D*, we estimated haplotype frequencies for each generation and each replicate:

$$A_1B_1 = D + p_{A1}p_{B1}$$

$$A_1B_2 = p_{A1}p_{B2} - D$$

$$A_2B_1 = p_{A2}p_{B1} - D$$

$$A_2B_2 = D + p_{A2}p_{B2}$$

To calculate the fitness of each haplotype, we aggregated all replicates from each generation to increase sample size for a more precise estimation and calculated overall haplotype frequency. In the insecticide treatment, the fitness of each haplotype compared to the control was calculated as simply the average haplotype frequency with insecticide divided by the average haplotype frequency in the control. For the control treatment, we estimated the fitness of each haplotype in the absence of insecticide over time by dividing the average haplotype frequency at generation F10 by the average haplotype frequency at generation F1. The fitness for each haplotype within a treatment was then normalized to the haplotype with the highest fitness, creating a measure of relative fitness.

Results

Kdr frequencies

The frequency of C1534 remained high over time in the insecticide treatment, starting at a frequency of 0.938±0.034 in generation F0 and ending in 0.965±0.025 in

generation F10 (Figure 3.1). Conversely, there was a significant decrease in the frequency of C1534 in the control treatment, which ended with a frequency of 0.860 ±0.044 in generation F10 (Chi-square= 8.6, p=0.003).

The frequency of I1016 increased non-linearly in the insecticide treatment from 0.595±0.056 in F0 to a peak at 0.945±0.032 in F7, and then decreased back to 0.808±0.045 in F10 (Figure 3.1). There was no change in the frequency of I1016 in the control treatment, which had a frequency of 0.595±0.057 in generation F10. However, the allele frequency was significantly higher in the insecticide treatment compared to the control (F10: Chi-square=33.4, p<0.001).

In the control treatment, all generations were in HWE for the 1016 locus (p>0.05), however 1534 was significantly out of HWE at generation F1 (F =0.53, p<0.001). In the insecticide treatment, all generations were out of HWE at both loci, as expected (p<0.001), except generation F3 was in HWE at 1016 (p=0.636).

The haplotype with both susceptible alleles, F1534/V1016, remained low in the insecticide treatment, starting at F1 with a frequency of 0.049±0.03 and ending at F10 with a frequency of 0.05±0.04 (Figure 3.2). However, in the control treatment, the frequency almost doubled, increasing from 0.07±0.04 at generation F1 to 0.12±0.03 in F10 (Chi-square=3.4, p=0.064). The F1534/I1016 haplotype was rare, remaining at a frequency close to zero for both treatments (0.007±0.003 for the control and 0.001±0.001 for insecticide treatment). The C1534/V1016 haplotype, on the other hand, remained at about 30% throughout the course of the experiment in the control treatment, decreasing slightly from a frequency of 0.34±0.10 in F1 and ending at 0.28±0.08 in F10 (Figure 3.2). Its frequency was significantly lower in the insecticide treatment at both F1 (Chi-

square=20.4, p<0.001) and F10 (Chi-square=17.8, p<0.001), yet didn't change over time, starting at 0.15±0.08 in F1 and ending at 0.14±0.10 in F10. Finally, the haplotype with both resistant alleles, C1534/V1016, was significantly higher in the insecticide treatment compared to the control throughout the course of the experiment (at F10: Chi-square =41.8, p<0.001), yet was unchanging over time in both treatments, remaining at a frequency of 0.60±0.07 in the control treatment and 0.82±0.11 in the insecticide treatment (Figure 3.2).

The haplotype with the highest fitness in the insecticide treatment was C1534/I1016, which has both resistant alleles (Table 3.1). However, in the control treatment, the haplotype with the highest fitness was F1534/V1016, which has both susceptible alleles. The fitness of F1534/I1016 was functionally zero in both treatments, as it was rarely detected. In the insecticide treatment, the fitness of C1534/V1016 was only 23% of the fitness of the haplotype with both resistant alleles, yet the haplotype with both susceptible alleles was slightly more fit than C1534/V1016. In the control treatment, the fitness of C1534/V1016 was about the same as C1534/I1016, which was half that of F1534/V1016.

Phenotypic resistance

The mean proportion of knocked-down mosquitoes in the initial population (F0, before selection) was 0.14 ± 0.13, which did not change over the course of the 10 generations of selection with insecticide (Figure 3.3). The insecticide-selected population had a mean knock-down proportion of 0.12 ± 0.18, while the control was 5.8 times more susceptible, displaying a knock-down proportion of 0.70 ± 0.14 (Welch t-test; t=-5.72,

df= 7.6, p-value = <0.001). Additionally, when eggs of F11 of the control treatment were placed into the insecticide tent and allowed to emerge, 100% of the adults died, confirming susceptibility.

Discussion

The two ways we evaluated the presence of selection in our experiment were (1) through a test of HWE, and (2) a change in allele frequencies over time. As expected, we found that both alleles were in HWE in the control treatment, suggesting that none of the assumptions of HW were violated, and specifically that selection was not occurring during the experiment. In I1016, this observation was supported by no overall change in the frequency, further suggesting no selection on the allele in the control treatment. The one exception was that generation F1 of C1534 in the control treatment was significantly out of HWE, indicating a violation of one of the assumptions during that initial generation. Given that it was a large, closed population that was mating at random, we conclude that selection is occurring. One hypothesis for selection on F1 in C1534 in the control treatment is that the initial population, F0, was a field-derived population that likely experienced heavy insecticide selection pressure. Placing their offspring into an environment without insecticide would impose a selection pressure if there is a fitness cost to the allele in the absence of insecticide. Indeed, we observed a decrease in the C1534 allele over the course of ten generations without insecticide, further suggesting a fitness cost to the allele.

The relative fitness estimates of the haplotypes with and without insecticide give additional insight into the individual allele fitness, providing further evidence of a fitness cost to C1534. As expected, in the control treatment, the haplotype with the highest

fitness was the one with both susceptible alleles, F1534/V1016. The C1534/V1016 haplotype, however, only had a fitness of one half that of the fully susceptible haplotype, indicating a cost to C1534. Curiously, the fitness of the haplotype with both resistant alleles, C1534/I1016 had a slightly higher fitness than C1534/V1016, suggesting that the I1016 allele may carry a small fitness advantage regardless of treatment. However, the F1534/I1016 haplotype displayed a fitness of zero in both treatments, indicating that either (1) I1016 is quite costly, or (2) the mutations are sequential in nature, with I1016 occurring only after the presence of C1534. Vera-Maloof et al. (25) also found a near absence of the F1534/I1016 haplotype in a linkage disequilibrium analysis of *kdr* mutations in field-caught Mexican *Ae. aegypti* populations, and similarly concluded that the evolution of the mutations are likely sequential.

Perhaps the most interesting finding is that the populations in the control treatment became phenotypically susceptible over the course of ten generations, yet the frequency of both *kdr* alleles remained high. This begs the question: are the *kdr* mutations responsible for phenotypic resistance to pyrethroids? Both the I1016 and C1534 *kdr* mutations are well established in the literature as significantly associated with pyrethroid resistance (7, 8, 22, 26), and the authors of one review study even say that "we can unequivocally state that the *kdr* genotype is an important – although, certainly, not necessarily the only – predictor of resistance phenotype" (27). What is key in that statement is that *kdr* is not the only genetic mechanism underlying resistance. Saavedra-Rodriguez et al. (28) used quantitative trait loci mapping to determine areas associated with permethrin resistance in *Ae aegypti*, and found that while I1016 was the largest contributor to the variance in *kdr* phenotype (~58.6%), various other loci were

responsible as well, including those involved with metabolic detoxification of insecticide. As molecular techniques for assessing metabolic mechanisms of resistance have become available, studies have found an overexpression of mixed-function oxidases associated with a deltamethrin-selected *Ae. aegypti* (18) and an upregulation of cytochrome P_{450} genes in permethrin-selected strains (29). Based on our results, it is likely that metabolic resistance was largely responsible for resistance in our populations and was subsequently lost in the control treatments, suggesting a fitness cost to those mechanisms as well, though further research is needed to explore this hypothesis.

Regardless of the underlying mechanisms, we did find a significant shift towards phenotypic susceptibility in the control populations, giving promise to the restoration of susceptibility in the field. While the populations did not technically meet the criteria for susceptibility defined by the WHO, which is less than 80% knock-down (21), the significant loss of resistance in only ten generations suggests that a longer duration without insecticide pressure could reduce resistance in the populations to the susceptibility threshold. Only two other studies have evaluated the loss of pyrethroid resistance in *Ae. aegypti* in the absence of insecticide. One study only looked at the change in frequency of the I1016 *kdr* mutation and found that the frequency dropped from 0.75 to 0.20 after 15 generations (17), yet did not assess the resulting phenotypic resistance. While their results are contrary to what we found, they performed their experiments on lab-derived strains that have a different genetic background to field strains, which could account for the differences in results. Chang et al. (30) performed the other study to assess loss of resistance in *Ae. aegypti*, and they found that after 15

generations in the absence of insecticide, the phenotype of the formerly permethrin-resistant population approached that of the susceptible strain, supporting our findings.

Overall, our results give compelling evidence of a fitness cost to the C1534 mutation and show that susceptibility can be restored into a highly permethrin-resistant *Ae. aegypti* population given an absence of insecticide pressure. These results support a vector control strategy that rotates chemicals in time and/or space to control pyrethroid resistant populations, providing areas where resistant populations can revert to susceptibility while still using an effective chemical to suppress overall population abundance (31). Future research should further investigate the fitness costs of resistance and potential mechanisms to accelerate the restoration of susceptibility.

Figures and Tables

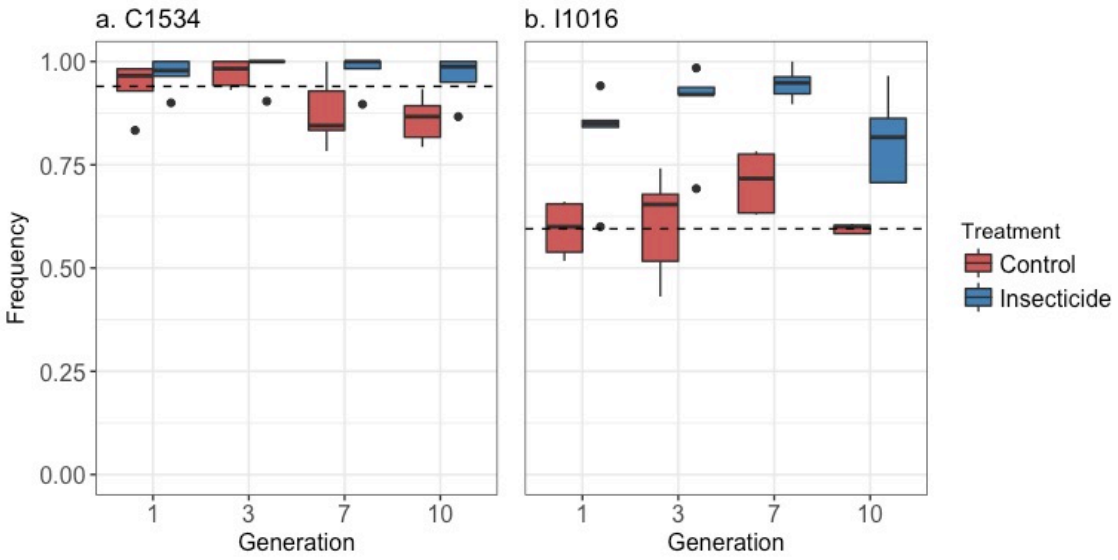

Figure 3.1: Allele frequencies over time in the insecticide and control treatments. The starting frequency is marked with a dotted line.

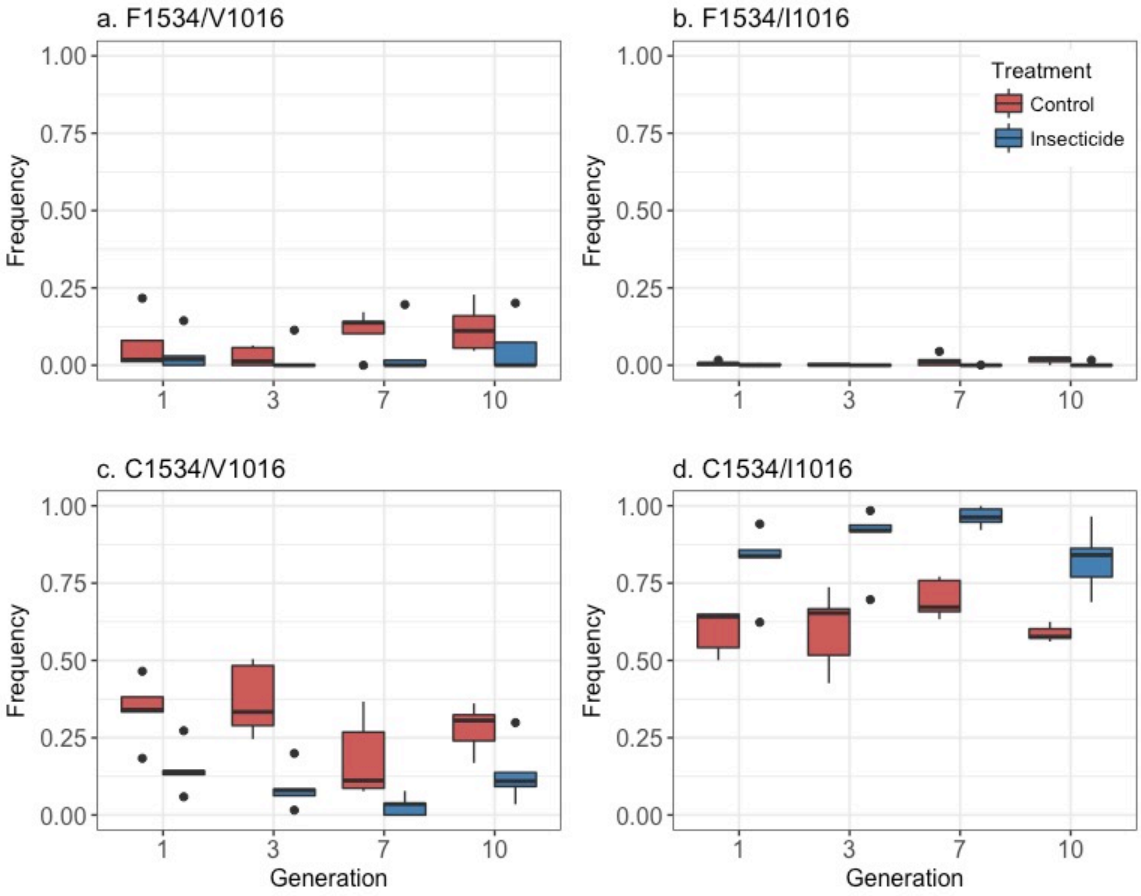

Figure 3.2: Haplotype frequencies over time in the insecticide and control treatments.

Table 3.1: Relative fitness of each haplotype in either the insecticide treatment or the control. In the insecticide treatment, relative fitness is the ratio of haplotype frequencies in the insecticide treatment to those in the control. In the control, relative fitness is the ratio of haplotype frequencies in generation F10 to those in F1

	F1534/V1016	F1534/I1016	C1534/V1016	C1534/I1016
Insecticide	0.37	0	0.23	1
Control	1	0	0.48	0.57

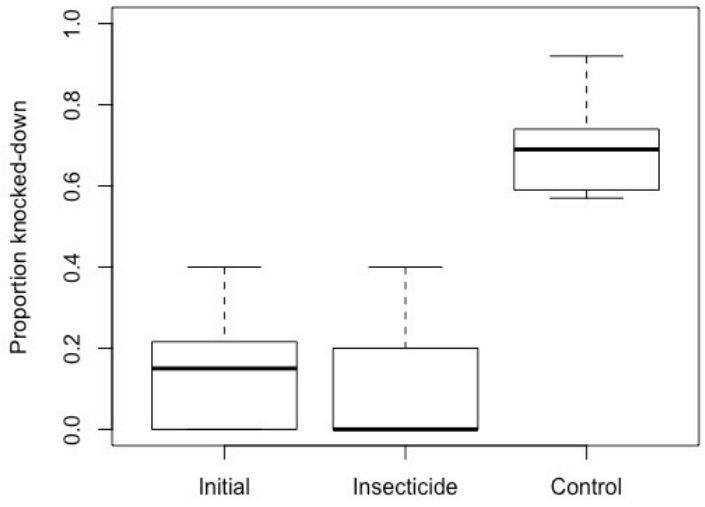

Figure 3.3: Phenotypic resistance. The proportion knocked-down at the diagnostic time of 30 minutes for the initial population (F0) and the two treatments at F10.

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Conclusion

Summary

The overall goal of this research was to understand ecological mechanisms impacting the evolution of pyrethroid resistance in field populations of *Ae. aegypti*. In Chapter 1, I described the patterns of *kdr* allele frequencies over space and time, and showed that frequencies are best analyzed at the fine spatial scale of the city block. Assessing *kdr* frequencies at a coarser spatial scale, which is what the majority of previous studies have done, obscured significant differences seen between the blocks. More importantly, the *kdr* allele frequencies varied throughout the year, decreasing during the dry season, when there is less insecticide application. This pattern suggests that there may be a fitness cost to resistance in the absence of strong selection pressure.

Chapters 2 and 3 provided evidence to support a fitness cost to pyrethroid resistance in *Ae aegypti*. In Chapter 2, I conducted an experiment to quantify various life history traits including development time, immature survival, and fecundity in susceptible and resistant populations of *Ae. aegypti* under high and low density treatments. Because *Ae. aegypti* are container-breeders, it is important to understand how density-dependent competition impacts these fitness parameters. I found that the resistant population laid fewer eggs than the susceptible population in the first gonotrophic cycle, regardless of density, indicating a fitness cost. The resistant population also had a longer development time, though only in the high density treatment. Most importantly, I found that density could mediate the resistance phenotype and genotype: under increased competition in the high density treatment, the adult mosquitoes that emerged were no longer phenotypically resistant, and the allele frequency of C1534 *kdr* mutation dropped significantly.

Chapter 3 provided additional evidence of a fitness cost to C1534 only, and showed that susceptibility can be restored into a resistant *Ae. aegypti* population in a relatively short timeframe in the absence of insecticide. Over the course of ten generations without insecticide pressure, there was a decline in the C1534 allele yet no change in I1016, which is the same pattern seen in Chapter 2 in the resistant population at high density. Furthermore, adults in generation F11 became significantly more phenotypically susceptible to the diagnostic dose of permethrin, measured with the CDC bottle bioassay. This demonstrates that susceptibility can be restored, even if the frequency of the *kdr* mutations remain high. These findings give compelling evidence for the success of vector control strategies that vary insecticide application in time and/or space, such as the rotation of insecticides.

Future research

One critical question that emerged from this research is how to accurately measure and predict the resistance phenotype. There are two current methods: (1) Resistance assays, such as the CDC bottle bioassay and the WHO resistance assay, and (2) by using molecular markers, such as *kdr* or genes that confer metabolic resistance. In Chapter 2, the results demonstrated that the CDC bottle bioassay gave two very different pictures of phenotypic resistance for the same population depending on the density in which the larvae were reared. If the larvae were reared at high density, the population appeared susceptible, with over 90% knocked-down at the diagnostic time, yet if the larvae were reared at low density, the resulting mosquitoes were diagnosed as resistant, with only 50% of the population knocked-down. These findings suggest that we cannot

rely on the current CDC bioassay to characterize field populations as “resistant” or “susceptible.”

Furthermore, both the current WHO (1) and CDC (2) phenotypic resistance assay protocols state that only non-bloodfed females of age 2-5 days should be used for the assays, yet those are not the individuals that transmit disease. For example, the extrinsic incubation period for dengue is 8-12 days (3), meaning that only females who are at least 8 days old and have had a blood meal can transmit the disease. The protocols state that the resistance phenotype can change based on age, sex, size, and feeding status of the mosquito, yet it is standard not to vary these traits during testing (1, 2). While the protocols are ensuring standardization, they are using a baseline that is not epidemiologically important, and they certainly are not giving a complete picture of resistance seen in the field. Research into the effect of these mosquito characteristics on the resistance phenotype is critically needed.

The other marker of resistance that is commonly used for *Ae. aegypti* is *kdr*. As discussed in Chapter 3, *kdr* may not be a good predictor of phenotypic resistance, either. In Chapter 3, the mosquito populations that lost phenotypic resistance to permethrin over ten generations without insecticide did not exhibit a significant decrease in either *kdr* mutation. Based on *kdr* mutations alone, all populations at the end of the experiment would have been characterized as resistant since their frequencies did not significantly change. While there are studies that demonstrate the relationship between *kdr* and phenotypic resistance (see reference (4) for a review), it appears that a high frequency of *kdr* can still be found in phenotypically susceptible mosquitoes. For example, Deming et. al (5) found a significant association between *kdr* and the resistance phenotype in five

different populations from the Yucatan, Mexico, yet the frequency of the C1534 *kdr* allele was as high as 0.94 and the I1016 as high as 0.64 in susceptible mosquitoes. Therefore, it may not be appropriate to rely on *kdr* alone as a marker of resistance.

It is clear that a new measure of resistance is needed. Molecular techniques, especially those that quantify the expression of genes involved in metabolic detoxification, are costly, time-consuming, and involve identifying multiple genes (6). Moreover, from a public health perspective, the phenotype is more important to characterize since that will ultimately determine the effectiveness of vector control activities. Therefore, further research should concentrate on understanding additional forces that affect phenotype, including ecological factors. This dissertation assessed a few ecological factors that have an impact on phenotype, such as intraspecific competition and life history traits, yet there is much left to understand. A more comprehensive knowledge of resistance ecology and evolution is key to effective vector and disease control.

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