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Spatial heterogeneity of insecticide resistance in the dengue vector *Aedes aegypti* in the Yucatan peninsula of Mexico presents unique vector control challenges

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An abstract of
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Abstract

Spatial heterogeneity of insecticide resistance in the dengue vector *Aedes aegypti* in the Yucatan peninsula of Mexico presents unique vector control challenges

By Regan Lee Deming

Background: The emergence of insecticide resistance in vector populations around the world threatens disease prevention and control. Dengue viruses, spread primarily by the *Aedes aegypti* mosquito, cause significant morbidity and mortality. Dengue is controlled mainly through insecticide application for vector control since there are no preventative medications or vaccines. Understanding how insecticide resistance moves through a population, its geographical distribution and the biological mechanisms underlying resistance is essential for effective evidence-based control programs.

Objective: This cross-sectional study is an initial assessment of insecticide resistance in *Ae. aegypti* mosquitoes across previously unstudied dengue-endemic towns in the Yucatan peninsula of Mexico. The study quantified the entomologic profile of these towns, phenotypic resistance and the frequency of two known molecular markers of resistance to certain pyrethroid insecticides.

Methods: Entomological surveys of *Ae. aegypti* were conducted in 5 towns in Yucatan, Mexico from June-August 2013. Emerged F_0-F_2 adults from eggs collected in each town were exposed to insecticides; deltamethrin, bendiocarb and chlorpyrifos and tested for resistance. Individuals tested against deltamethrin were also evaluated for the presence of two kdr mutations known to be associated with pyrethoid resistance, V1016I and F1534C, to assess the validity of these as molecular markers for deltamethrin resistance.

Results: CDC Bottle Bioassay tests showed high levels of resistance to deltamethrin and chrlorpyrifos, and limited resistance to bendiocarb, with variations between study towns. Frequencies ranged between 0.47-0.74 for the V1016I mutation and showed a highly significant association with deltamethrin resistance phenotype in each town ($p < 0.002$). Frequencies ranging between 0.59-0.94 were found for the F1534C mutation, showing significant association with deltamethrin resistance phenotype in 3 of the 5 towns, ($p = 0.01$, $p < 0.0001$ and $p < 0.0001$).

Discussion: These data have identified heterogeneity of insecticide resistance in previously unstudied populations and offer key insights into the development of these patterns. Several driving forces lead to development of insecticide resistance in mosquito populations, including pressure from insecticide application as well as human behavior and movement patterns. Heterogeneity of resistance patterns over a small geographical area poses a challenge to vector control programs, as employing interventions at a small scale is not always feasible.

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Background

Dengue is the most important and widespread mosquito-borne viral infection of humans in the world [1]. Roughly 70-100 million cases of dengue virus (DENV) infection occur per year throughout the tropical and subtropical world. It is estimated that up to 55% of the world's population is at risk of infection in 128 countries, where 824 million people live in urban environments [2]. In the last twenty years, dengue fever epidemics have increased in both number and magnitude, mainly due to a range expansion of the *Aedes aegypti* mosquito, the primary vector of dengue viruses [3, 4]. Increasing trends in the distribution of the world's population in urban centers and the globalization of human movement patterns has facilitated the expansion in habitat and abundance of *Ae. aegypti* and contributed to the global re-emergence of dengue [1, 5, 6].

There are four known circulating serotypes of DENV, denoted DEN-1, DEN-2, DEN-3 and DEN-4 [7]. Although most dengue infections are subclinical, symptomatic cases can include fever, malaise, and musculoskeletal pain. An estimated 390 million [8] human infections of dengue occur each year and of these approximately 500,000 progress to the most severe manifestations of DENV infection: Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS), which can be fatal in the absence of appropriate hospital treatment [9]. Although dengue can cause high morbidity and even death, infections are often inapparent, meaning there are no clinical symptoms, and therefore go undetected. Several studies have been undertaken to estimate the number of inapparent cases compared to those resulting in febrile illness or other dengue fever symptoms. Evidence suggests that there is strong variation in the ratio of symptomatic-to-inapparent (S:I) cases on both a focal geographic level and over time [10]. Results from a study in Nicaragua showed S:I ratios as low as 1:18 after one year of observation, which increased

to 1:3 after 3 years [11]. Variation was also observed in a study in Thailand, where the S:I ratio was 2:1 one year and 1:9 in another year [12].

Many factors have influenced the re-emergence and spread of dengue, among them globalization and related trends including population growth, unplanned urbanization, air travel and other transport of people, animals and merchandise [6]. The *Ae. aegypti* mosquito is highly adapted to the human environment; it breeds in artificial containers found in and around the home [13], it prefers to rest indoors [14], it rarely disperses beyond 100 meters from its breeding habitat [15], and it feeds frequently and preferentially on humans, particularly those who spend more time in their homes [16, 17].

There are currently no therapeutic medications or vaccines for dengue. Vaccines are being researched, but the existence of multiple serotypes has made development of an effective vaccine difficult [8, 9, 18, 19]. The most promising vaccine is currently in phase 3 of development, and results from phase 2b showed a 30.2% efficacy overall but with varying degrees of efficacy against individual serotypes [20]. In the meantime, alternative methods of control are necessary to both prevent and decrease the severity of the disease [21, 22].

Vector control of *Ae. aegypti* presently serves as the only effective approach for preventing dengue transmission. Primary vector control methods include environmental sanitation and source reduction through the elimination of artificial containers inhabited by immature mosquitoes and insecticide application including the addition of larvicides to breeding sites and ultra-low-volume (ULV) and indoor space spraying targeting adult mosquitoes [23, 24]. Another strategy showing promising efficacy for dengue vector control has been the use of insecticide treated materials. Due to the day-time biting

behavior of female *Ae. aegypti*, the use of insecticide treated bednets is not generally recommended, although they can reduce household-level *Ae. aegypti* infestations [25]. Other insecticide-treated materials such as window and door curtains and water storage container covers have been shown to be effective at reducing household-level *Ae. aegypti* infestations [26]. A study conducted by Vanlerberghe et al. [27] in Venezuela found significant declines in the number of adult and immature mosquitoes found in and around the houses over the course of an 18-month intervention with insecticide treated materials, even though less than 50% of the houses enrolled in the trial continued use of them throughout the duration of the study. Another study conducted in Merida, Mexico [28] also demonstrated that insecticide treated curtains could impact dengue vector populations, but on a more modest scale. This less substantial impact could potentially be due to the prevalence of insecticide resistant mosquitoes in Merida, Mexico [29].

Due to the epidemiology and ecology of dengue, vector control is necessary and insecticide application is widely used. Given the heavy reliance on chemical insecticides to control dengue vectors, resistance to those most commonly applied has risen dramatically in recent years. This is cause for alarm to those involved in dengue prevention, since resistance to insecticides can potentially compromise the predominant strategies for dengue vector control [30-32].

The emergence and spread of insecticide resistance in arthropod disease vectors was first reported by the World Health Organization (WHO) in 1976 when malaria eradication efforts showed resistance to DDT had developed in many of the malaria mosquito vectors [33]. The development of insecticide resistance can occur in vector insects through selection pressure placed on a vector population with regular insecticide

exposure. In an effort to combat resistance, vector control programs have switched to newer insecticides and more focal or targeted vector control, rather than 'blanket' applications, yet the problem continues to plague vector-borne disease prevention efforts [23, 34]**.** Currently, research on the molecular mechanisms of resistance is attempting to identify early markers of resistance pathways before they become established in the environment [35].

In practice, there are four classes of insecticides recommended for public health use and vector control by the WHO [36]: carbamates, organophosphates, organocholorines and pyrethroids. There are two principal mechanisms of resistance to these insecticides seen in *Ae. aegypti*: 1) metabolic (or enzymatic) resistance, which occurs through the overproduction of detoxifying enzymes that metabolize the insecticide, and 2) target site resistance, where mutations on the insecticide's target site within the mosquito prevent it from binding. An important target site resistance mechanism is known as 'knockdown resistance' (kdr), and results from point mutations on the sodium channel gene resulting in conformational changes that prevent the insecticide from binding to its target site. Kdr is associated with resistance to both DDT and pyrethroids, and inhibits the ability of the insecticide to initially 'knock down', and ultimately kill, the arthropod [8, 37].

Pyrethroids have become the most frequently and globally used public health insecticide due to their low cost and low toxicity to mammals, in addition to their residual properties on applied surfaces [38]. It is of considerable concern when kdr is found in wild populations of vector mosquitoes, given there are few suitable alternatives to pyrethroid insecticides approved for public health use. There are several point mutations

known to confer kdr-type insecticide resistance in *Ae. aegypti*. Although there can be other explanations for phenotypic resistance, research has shown that mutations on the 1016 (V1016I) and 1534 (F1534C) codons of domains II and III of the voltage gated sodium channel gene are strongly associated with kdr phenotype in *Aedes* mosquitoes [37, 39, 40]. Using molecular diagnostic tools to detect such mutations is the best available method for diagnosing kdr mutations and therefore an important component for monitoring resistance frequencies in mosquito populations [41].

Between 2007 and 2009, Siller et al. [42] reported the increase in frequency of the Ile1016 allele in several localities within the state of Veracruz, Mexico; the two most significant increases were in the towns of Veracruz (245.8%) and Martinez de la Torre (52%) while the smallest increase in frequency was 5.1% in the town of Panuco. Similar findings by Ponce Garcia et al. [29] showed over the course of 14 years, several Mexican states, including Veracruz and Yucatan, experienced a significant increase in the frequency of the V1016I allele. In Merida, Yucatan in particular, V1016I had not been detected when samples were first collected in 1999, but by 2007, a frequency as high as 54% was observed, although frequencies varied by sample location. A more recent study in the state of Guerrero, in southern Mexico, found V1016I at a frequency of 80% and all mosquitoes sequenced had the F1534C allele mutation as well [43]. The F1534C mutation was first reported in *Ae. aegypti* mosquitoes in Asia and first detected in North America on the Cayman Islands where results provided evidence that the mutation conferred resistance to pyrethroid insecticides [39]. The detection of the F1534C mutation in the State of Guerrero was the first report of the mutation in Mexico [43].

There are two possible explanations for such a rapid and dramatic increase in kdr resistance patterns. The emergence of kdr genotypes may have arisen independently due to ULV spraying and other widespread applications of insecticides. An alternative explanation is that the kdr genes were introduced through the immigration of mosquitoes from elsewhere with high kdr frequencies, via artificial containers containing eggs or adult mosquitoes carried in transport vehicles such as cars or buses [44, 45]. It is important to understand why and how resistance develops, as this can influence the planning of public health intervention strategies, particularly in countries such as Mexico where resistance has increased quickly and chemical insecticides are widely used as part of its dengue control strategy.

Mexico relies heavily on insecticides to control dengue and other vector-borne diseases. Since the 1950's, vector control programs in Mexico relied heavily on the use of the organochlorine insecticide DDT to control malaria and arboviral diseases such as yellow fever. In the 1980's and 1990's, Mexico switched to primarily using organophosphates for vector control and for the decade between 2000 and 2010 Mexico used second and third generation pyrethroid insecticides such as deltamethrin and cypermethrin as their primarily method for controlling adult mosquitoes [29].

Dengue outbreaks have occurred throughout Mexico since the first report of the virus with a single serotype, DENV-1, in 1979, followed by an outbreak of DENV-1 and DENV-4 in 1984, DENV-2 and DENV-4 in 1994 and finally an epidemic from 1995-1997 where all 4 serotypes were detected [46]. It is well documented that as viral genotypes or serotypes are displaced by the introduction of new types into the environment the number of severe cases, including DHF and DSS increases [47, 48].

The Yucatan State experienced a similar trend with the introduction of DENV serotypes, and reports show that as more serotypes were introduced into the state, the incidence of severe dengue, particularly hemorrhagic dengue, has also increased [28]. During 1984, an outbreak of DENV-4 in the Yucatan resulted in nine hemorrhagic cases reported, but only one of them met WHO criteria for a DHF case. In 2002, an outbreak of DENV-2 was the first report of the American-Asian genotype in Yucatan State, a genotype known to be associated with severe dengue. The outbreak in 2002 resulted in 282 confirmed dengue fever cases. Of the confirmed cases, 162 included hemorrhagic symptoms and 87 were confirmed DHF. The city of Merida is the capital and main urban center of Yucatan State, and dengue transmission is hyper-endemic. In recent decades, co-circulation of multiple serotypes and a high abundance of *Ae. aegypti* has increased the risk of severe dengue for the population living in and around the city center [28].

While the intensity of insecticide use for mosquito control in the city of Merida is relatively consistent, the surrounding communities have experienced more sporadic insecticide applications. Resistance to insecticides has been reported at varying degrees of frequency from the city of Merida, but no entomological data, including resistance data, are available from these surrounding towns (Che-Mendoza, personal communication). Understanding the spatial heterogeneity of insecticide resistance is important for the application and efficacy of vector control interventions. The overall goal of this study is to further understand the level of insecticide resistance in particular populations of *Ae. aegypti* and the frequency of two kdr mutations known to be associated with insecticide resistance.

The main aims of this study are:

Aim 1. Describe how entomological indicators of dengue transmission risk differ between 5 satellite towns of Merida, Yucatan, Mexico

Aim 2. Analyze the patterns of insecticide resistance and kdr gene frequency in the populations of *Ae. aegypti* in 5 satellite towns of Merida.

Aim 3. Determine how the history of insecticide use, human behavior, incidence of dengue cases and proximity to Merida are related to the prevalence and intensity of insecticide resistance in the 5 satellite towns.

Research Methods:

Study area

This study was conducted on the Yucatán peninsula of southern Mexico and included five towns located on the periphery of the state's capital, Merida. Merida is in a subtropical environment with a mean annual temperature of 33 °C, with mean temperatures ranging from 29 °C in December to 34 °C in July. The rainy season occurs from May to October, which overlaps with the peak dengue transmission season between July and October, although cases continue to occur throughout the year [49]. Dengue is highly endemic throughout the Yucatan peninsula, and vector control activities are widespread. Current vector control strategies include ultra-low volume (ULV) spraying with the organophosphate insecticide chlorpyrifos and indoor space spraying with pyrethroids and carbamates for adult mosquito control and temephos application for *Ae. aegypti* breeding site control (Che-Mendoza, personal communication).

Surrounding Merida are small, densely populated, satellite towns that are normally connected to Merida by a single road. The 5 towns selected for this study were located 15-35km from Merida's city center and at least 20km from one another. Although each town has their own municipal jurisdiction, including entities responsible for vector control, there is close connectivity with Merida (Figure 1).

Study design:

A cross-sectional, entomologic survey was performed in 250 houses across 5 satellite towns in close proximity to Merida, Yucatan, Mexico. The study towns of San Lorenzo, Acanceh, Progreso, Hunucma and Conkal are suburban municipalities located outside the urban center Merida and range in population size and distance to Merida (Table 1). Ten houses on five blocks in each of the five towns were sampled. Data for this study were collected from June-August 2013.

Entomologic collections

Beginning in late June 2013, two five-person entomology collection teams were provided a map of the five study towns indicating the five selected blocks to be sampled. Blocks were selected based on proximity to the town's central square, ensuring a selection of blocks with differing distances and direction from the central square or primary region of commerce (Figure 2). Ten houses on each block within the towns were sampled to survey *Ae. aegypti* breeding and adult mosquito infestation levels.

Households were surveyed between the hours of 8:00am and 2:00pm in sequence until ten houses from each block had been sampled. Indoor adult mosquito collections

were performed using Prokopack aspirators [50] for 10-15 minutes per household. Aspirator collections were attempted in each room of the house when authorized by the occupants, including walls, underneath furniture and counter space, and inside closets. All adult female *Ae. aegypti* captured inside the home were assessed for bloodmeal presence and the digestive state of the abdomen following Detinova classification of sella scores [51]**.** The mosquitoes were then desiccated and stored in individual tubes at -20 °C for future molecular analysis.

Breeding sites in and around the home were surveyed for the presence of mosquito larvae and pupae and were classified using WHO standards [52]. Larvae and pupae were collected from each positive breeding site and placed in plastic Whirl-Pak® sample bags (Bioquip, Rancho Dominguez, CA) and brought back to the field insectary where they were allowed to emerge and identified to *Aedes* species or *Culex* genus. All emerged adults identified as female *Ae. aegypti* were desiccated and individually vialed and labeled with the house number and date of collection.

On every sampled block, ovitraps were placed for several weeks for *Ae. aegypti* egg collection. Using the ovitrap design of Lenhart et al. [53], each ovitrap was a dark colored 5 liter bucket lined with a strip of white fabric labeled with the household, block, town, trap number and date. The ovitraps were placed in dark locations outside the home, protected from precipitation, where *Ae. aegypti* are likely to rest. Ovitrap fabric was checked weekly and those with eggs were dried and stored in sealable plastic bags. Desiccated adult mosquitoes and eggs were then transported back to the CDC laboratories in Atlanta, GA for molecular analyses

Household surveys

A short survey was administered to the occupants of the house regarding basic demographics, including the number of occupants in the house, water and insecticide use, and movement patterns, particularly movement frequency and transportation methods between the town of residence and Merida.

CDC Bottle Bioassays

The eggs collected from the ovitraps were hatched using a NAPCO E series, 5831 vacuum oven and reared to adults. Females were tested for insecticide resistance at the CDC insectaries using the CDC Bottle Bioassay protocol [54]. Bottle Bioassays were conducted by pooling reared mosquitoes from the 5 sampled blocks in each town. The five populations were evaluated against the insecticides deltamethrin, bendiocarb and chlorpyrifos using the suggested diagnostic doses (DD) and diagnostic times (DT) previously established for susceptible strains by the CDC [54] (Table 2).

Female *Ae. aegypti* mosquitoes that were 2-5 days old were exposed to bottles treated with the different insecticides according to the DD and DT (15-25 mosquitoes per bottle, four replicates per test). Tests were conducted on F_0-F_2 *Ae. aegypti* depending on the town they were collected from and the insecticide being tested. At the diagnostic time, the number of surviving and dead mosquitoes was recorded and the mosquitoes were separated, placed in individual tubes, and labeled according to the population, insecticide tested, and whether they were dead or alive following the test and then stored at -20 °C for future molecular testing.

Molecular assays

To assess the association of kdr genotype with deltamethrin resistant phenotype were tested against deltamethrin in the bioassays and genotyped for the V1016I and F1534C kdr alleles. All molecular assays were performed in the CDC laboratory in Atlanta, GA. DNA was extracted from a leg or other body part from each individual mosquito in a solution of 45 µl of H_2O and 5 µl of Promega Taq DNA Polymerase10x Buffer with $MgCl_2$ (Madison, WI) in a 96 well PCR plate. Samples were incubated at 95 °C in a BioRad icycler thermocycler for 15 minutes.

Polymerase chain reaction (PCR). When performing the PCR assays, DNA from the susceptible Rockefeller *Ae. aegypti* strain was used as a susceptible (wild-type) homozygote control. DNA from the MF5 strain was used as a heterozygote control for both kdr mutations. Real-time PCR was used to test for the V1016I and F1534C single nucleotide polymorphisms (SNPs) and results were analyzed based on the melting temperature curves. The PCR reaction for the V1016I allele consisted of 4 ul of iQ™ SYBR® Green Supermix (Bio-Rad 170-8880), 2 ul of each of the Val1016f, Ile1016f and Ile1016r primers, and1 ul of DNA template [37]. The PCR reaction for the F1534C allele consisted of 7.67 ul of iQ™ SYBR® Green Supermix (Bio-Rad 170-8880),1 ul each of the Phe1534+f and Phe1534+r primers and 0.33 ul of the Cys1534+f primers, and1 ul of DNA template [55].

Primer	Primer sequence 5'-3'
Val1016f	GCGGGCGGCGGGGGCGGGGCCACAAATTGTTTCCCACCCGCACCGG
Ile1016f	GCGGGCACAAATTGTTTCCCACCCGCACTGA
Ile1016r	TGATGAACCSGAATTGGACAAAAGC.
Cys1534+f	GCGGGCAGGGCGGCGGGGGCGGGGCCTCTACTTTGTGTTCTTCATCATGTG
Phe1534+f	GCGGGCTCTACTTTGTGTTCTTCATCATATT
Phe1534+r	TCTGCTCGTTGAAGTTGTCGAT

Reaction conditions for detection of the V1016I allele were: 95 ° C for 3 min, 40 cycles of 95 ° C for 10 sec, 60 ° C for 10 sec, 72 ° C for 30 sec, and a final hold of 95 ° C for 10 sec. To calculate the melting curve, the final PCR product was heated from 65 ° C to 95 ° C at 0.2 ° C increments every 10 sec. Reaction conditions for the detection of the F1534C allele were: 95 ° C for 3 min, 40 cycles of 95 ° C for 10 sec, 57 ° C for 10 sec, 72 ° C for 30 sec, and a final hold of 95 ° C for 10 sec. To calculate the melting curve, the final PCR product was heated from 65 ° C to 95 ° C at 0.5 ° C increments every 5 sec. Real-time PCR reactions were carried out in a BioRad CFX96 Real-Time System C1000 thermal cycler. Results were read using Precision Melt Analysis Software ™.

For the V1016I mutation, there are 3 potential melting peak profiles. A peak at 79^{o}C corresponds to Isoleucine and a peak at 85^{o}C corresponds to Valine (wild type). Individuals with peaks at both 79^{o}C and 85^{o}C are considered heterozygotes for that

mutation (Figure 4a). For the F1534C mutation there are 3 potential melting peak profiles. A peak at 80^{o}C corresponds to Phenylalanine (wild type) and a peak at 85^{o}C corresponds to Cysteine. Individuals with peaks at both 80^{o}C and 85^{o}C are considered heterozygotes for that mutation (Figure 4b).

Insecticide application and reported dengue cases

Data from the Mexican Health Secretariat (Secretaria de Salud) Epidemiological Platform for Dengue Surveillance [56] was extracted and analyzed for the five towns during the epidemiological weeks 1-35 of 2013 (January 1-September 1). These were the most current data regarding insecticide application and reported dengue cases preceding and during sample collections. Using the extracted data, the type of insecticide applied through indoor space spraying when dengue cases were reported was summarized for all five towns.

Data management and analysis

Descriptive analyses using the WHO entomological surveillance guidelines were used to determine the geographical distribution of vectors across the five towns [57]. The indices used were the house index (HI; the percent of houses with larvae and/or pupae), the container index (CI; the percent of water holding containers infested with larvae or pupae), and the Breteau index (BI; the number of positive containers per 100 houses inspected). Additional indices calculated were the adult index (AI; the average number of adult female *Ae. aegypti* per house), the average number of people per house by town (calculated by averaging the number of people reported by the respondent to be living in

the house across the entire town), and the average number of adult *Ae. aegypti* per person (calculated using the AI and the average number of people per house). Analysis of mortality from each of the three insecticides was obtained for each town based on the CDC recommended DT and DD (Table 2). Populations were classified as resistant or susceptible to tested insecticides using the WHO guidelines for interpreting mortality measures [58]. They are as follows; mortality in the range 98-100% indicates susceptibility, mortality between 90-97% suggests resistance genes should be investigated in the population as resistance may be developing, and mortality less than 90% confirms resistance if the results are from >100 mosquitoes.

The allele frequency for the V1016I and F1534C mutations were calculated using the equation:

$$\frac{(\text{n heterozygotes} + 2(\text{n homozygotes})}{2(\text{total n mosquitoes analyzed})}$$

The 95% confidence interval (CI95) around the frequency of each of the alleles was calculated using a Wald interval [29]. Fisher's exact tests were performed in SAS 9.3 to test the association of each genotype with phenotypic resistance or susceptibility.

Results

Entomological Indices

Over the course of the study, 249 houses had entomological surveys performed. By town, the HI ranged from 10-54%, the CI ranged from 3.8-25.7%, the Breteau Index ranged from 14-102 and the AI ranged from 1.12-3.24 mosquitoes per house. San Lorenzo had an average of 4.1 (SD =1.5) people per house, Acanceh had an average of 4.7 (SD = 1.9) people per house, Progreso had an average of 3.7 (SD = 1.9) people per house, Hunucma had an average of 4.8 (SD = 2.0) people per house and Conkal had an average of 4.2 (SD = 2.2) people per house. In each house, the number of adult female *Ae. aegypti* per person ranged from 0.3-0.8 (Table 3).

Of the five communities, four had 20 5-liter ovitraps placed for 2 or 3 weeks, the exception being San Lorenzo which had 3 16oz ovitraps at 20 houses the first week and at 40 houses the second week. The majority of traps were positive for 1 or more eggs (Figure 5a). Week one showed low egg counts with an average number of eggs collected per positive ovitrap ranging from 26.8-33.4 in each town, week 2 had an average egg count per positive ovitrap ranging from 81.3-273.9 and only two towns, Progreso and Hunucma, had an average egg count of 280.1 and 431.6 per positve ovitrap, respectively, for week 3 (Figure 5b).

A total of 562 adult female *Ae. aegypti* were collected inside the houses across the five towns and assessed for physiological stage of bloodmeal digestion. The proportion of bloodfed mosquitoes was analyzed by community; in San Lorenzo 42% (68/162), Acanceh 71% (77/108), Progreso 60% (37/62), Hunucma 74% (91/123) and Conkal 57% (61/107) were recorded as bloodfed (Table 4).

CDC Bottle Bioassays

A total of 15 CDC Bottle Bioassays were performed; 402 female *Ae. aegypti* were tested using chloropyrifos, 359 were tested using bendiocarb and 429 were tested using deltamethrin. The development of resistance to bendiocarb was only detected in 1 of the 5 communities, Progreso (95% mortality), while all other communities expressed complete susceptibility (100% mortality) per WHO criteria. Resistance to chlorpyrifos was observed in all 5 communities at differing levels, ranging in mortality from 19.1% in San Lorenzo to 55.9% mortality in Hunucma. Resistance to deltamethrin also varied between the communities; San Lorenzo showed the lowest mortality rate at 62.7% while Conkal showed the highest mortality rate at 88.1% (Figures 3a-3c).

Kdr Genotyping

DNA was extracted and PCR was performed on 422 of the 429 mosquitoes tested against deltamethrin in the CDC Bottle Bioassay. Among all mosquitoes genotyped for the V1016I mutation (n=422), 13.3% (n=56) were wild-type (susceptible) homozygotes (SS), 52.8% (n=223) were heterozygotes (SR) and 33.9% (n=143) were homozygous resistant (RR). Among all mosquitoes genotyped for the F1534C mutation, 5.5% (n=23) were SS, 36.0% (n=152) SR and 58.5% (n=247) RR. The Fisher exact test showed a highly significant association between the 1016I genotype and deltamethrin resistance in all 5 communities: San Lorenzo ($p < 0.0001$), Acanceh ($p < 0.0001$) Progreso ($p = 0.002$) Hunucma ($p < 0.0001$) and Conkal ($p < 0.0001$). A significant association between

deltamethrin resistance and the 1534C genotype was seen in 3 of the 5 communities: San Lorenzo ($p= 0.01$) Acanceh ($p <0.0001$) and Hunucma ($p <0.0001$) (Table 5).

Of the mosquitoes resistant to deltamethrin, 99.9% (n=84/85) were positive for 1016I and 82.4% (70/85) were homozygous resistant (RR). Among the resistant mosquitoes, 99.9 % (n=84/85) were positive for 1534C and 97.6% (83/85) of the resistant mosquitoes were RR. There were also 140 mosquitoes homozygous for both resistant genotypes but only 50.0% (n=70/140) of these were resistant in the bottle bioassays. 97.9% (n=140/143) of 1016I RR individuals and 56.7% (n=140/247) 1534C RR individuals were double homozygotes.

1016I appeared in all of the five towns with an overall frequency of 60.3% ($\pm$4.67 CI95); San Lorenzo had a frequency of 73.5% (CI $\pm$9.5), Acanceh 49.4% (CI $\pm$10.6), Progreso 70.0% (CI $\pm$9.9), Hunucma 47.7% (CI $\pm$10.4), and Conkal 60.8% (CI $\pm$10.4). 1534C also appeared in all of the five towns at an overall frequency of 76.5% (CI $\pm$4.0); San Lorenzo had a frequency of 93.9% (CI $\pm$5.1), Acanceh 67.1% (CI $\pm$10.1), Progreso 95.6% (CI $\pm$4.1), Hunucma 59.1% (CI $\pm$10.2), and Conkal 65.8% (CI $\pm$10.2) (Table 6).

Household Surveys

Daily use of motorized transportation to travel within and outside of town was reported throughout the towns. Hunucma reported the lowest use of daily transit, with only 40% (20/50), while San Lorenzo reported the highest, with 65% (32/49) of those surveyed using motorized transit daily (Table 7a). The type of motorized vehicle and most common type of transportation varied across the study sites. In San Lorenzo the most common type of transport was bus (61%; 30/49), while in Acanceh the most common

type of transport was short distance motorcycle taxi (52%; 26/50). In Progreso, two types of transport were identified as the most common: car (34%; 17/50) and bus (36%, 18/50). Residents of Hunucma identified motorcycle taxis and bicycles as the most common types of transit (both 28%; 14/50) and in Conkal, cars and motorcycle taxis were the most common (26%; 13/50, and 24%; 12/50, respectfully) (Table 7b). When questioned about the purpose for traveling outside of their towns, the respondents most commonly reported traveling for shopping and work (Table 7c).

Findings from the survey also showed regular insecticide use inside the homes across in towns; San Lorenzo 90% (44/49), Acanceh 86%, Progreso 84% (42/50), Hunucma 78% (39/50) and Conkal 86% (43/50).

Insecticide application and dengue cases by town

Data from the Mexican Health Secretariat showed that most houses reporting dengue cases during the first 35 epidemiological weeks of 2013 were treated with indoor space spraying using propoxur insecticide. Progreso had the greatest number of cases during this time with 88, Uman* reported 52, Hunucma reported 49, Acanceh reported 19 and Conkal reported 8 (Table 8).

*Uman-San Lorenzo is a neighborhood part of the municipality of Uman. Dengue cases are reported on the municipal level.

Discussion

The main objective of this study was to characterize the insecticide resistance status of *Ae. aegypti* and assess the level of insecticide resistance heterogeneity within and between small communities outside of Merida, Mexico. The entomological surveys showed the presence of high numbers of both immature and adult mosquitoes in the home environments of all five communities. The level of heterogeneity in both resistant phenotypes and genotypes between these towns illustrates the complexity and focal nature of insecticide resistance, even at a small geographic scale. Further, this study confirmed the association between two kdr mutations and phenotypic resistance to deltamethrin insecticide [39]. Additionally, several factors were investigated that could be related to insecticide resistance and how it emerges in populations, including the frequency and type of human movement patterns and the application of insecticides by the state vector control program and occupants in and around homes. Due to the cross-sectional nature of this study, temporal correlations are unable to be made.

Adult and immature indices were not compared between communities due to variation in the times data were collected. Data collection began in late June, when daily heavy rains had not yet begun and mosquito productivity remained low. Final data collections took place in mid-August when it rained daily and mosquito populations were high. The impact of this sampling scheme was most apparent from the ovitrap egg yields, as the number of positive traps was consistent throughout the data collection period for all towns, but the number of eggs per positive ovitrap consistently increased as the rainy season progressed.

The presence of a bloodmeal and the stage of bloodmeal digestion (Sella score) can be used to approximate the age of a mosquito and therefore the level of vectorial capacity it may have in the environment. [51]. Many of the mosquitoes collected during indoor aspiration did not show evidence of a recent bloodmeal, but in each town, excluding San Lorenzo, more than half of the collected mosquitoes showed indications of a previous bloodmeal. These results could be an indication of an older mosquito population and therefore a higher risk of infection in these towns.

Low mortality was observed in all populations tested for chlorpyrifos, suggesting high resistance to this insecticide across the region. Interestingly, susceptibility to the carbamate insecticide bendiocarb was high in all communities except Progreso, where mortality was 95% (suggesting incipient resistance). At the time immediately preceding and during this study, Progreso had reported the greatest number of dengue cases out of the 5 study towns, and as such, had received the most insecticide spray treatments by the vector control program using the carbamate insecticide propoxur. This may have contributed to the observed decreased susceptibility to bendiocarb. Organphosphate and carbamate insecticides (the insecticide families for chlorpyrifos and bendiocarb, respectively) often share the same target site for insecticide resistance. However, carbamate resistance was only detected in Progreso while chlorpyrifos resistance was widespread. Elevated levels of insensitive acetylcholine-esterase (AChE) have been shown to be associated with insecticide resistance for both organophosphates and carbamates, and mutations on the ace-1 gene often confer cross resistance to both insecticide groups [59, 60]. However, in this case, the observed resistance is likely due to an alternative mechanism since cross-resistance was not observed.

Mortality data from the deltamethrin bottle bioassay showed significant associations with the presence of both kdr mutations (1016I and 1534C). However, not all mosquitoes containing a mutant allele were phenotypically resistant. The 1016I mutation is known to be associated with resistance to type I and II pyrethroids (including deltamethrin), as well as DDT [37] while the 1534C mutation is known to be associated most strongly with resistance to type I pyrethroids such as permethrin, as well as DDT [39]. Although the frequencies of both mutations were high in deltamethrin resistant individuals, the alleles were still present in many susceptible individuals. In addition, not all mosquitoes resistant in the bioassays contained the resistant alleles. This suggests that multiple resistance mechanisms could be contributing to the resistant phenotypes, including metabolic mechanisms arising from the overproduction of detoxifying enzymes known to confer pyrethroid resistance [61]. Investigation into these mechanisms and the role detoxifying enzymes may have on resistance in these populations will help to further explain the resistance patterns observed.

A high percentage of mosquitoes classified as homozygous RR for one kdr mutation contained both mutations or were double homozygous RR. This trend was stronger in those homozygous for 1016I than those homozygous for 1534C. Interestingly, of the double homozygous RR individuals, there was an equivalent number that were classified as phenotypically resistant or susceptible. Some evidence suggests that pyrethroid resistance has an associated fitness cost resulting in reduced larval development and adult longevity [62]. Perhaps these double homozygous individuals obtain a fitness advantage while still maintaining a certain degree of resistance.

The decision to focus on resistance to deltamethrin was made due to the historic use of pyrethroids and the current insecticide application strategy in the Yucatan. From 1998 to 2009, pyrethroids, were the primary insecticides used for adult mosquito control and deltamethrin was the primary insecticide used for indoor space spraying when a dengue case was reported. Starting in 2010, evidence suggesting high resistance to pyrethroids and a high frequency of the 1016I mutation in the population [43] convinced vector control authorities to modify their strategy. Since 2010, up until when collections for this study were done in 2013, carbamates (bendiocarb, and later propoxur) have been predominantly used for indoor space spraying although deltamethrin still continues to be used. The results from this study show that after a switch away from pyrethroids for over 3 years, phenotypic resistance and a high frequency of kdr alleles still remain in the vector population. The strategy for vector control will be changing again in 2014, as deltamethrin will again be used as the principal insecticide for indoor space spraying (Che-Mendoza personal communication). In light of the findings from this study and these new control plans, it will be important to closely monitor the efficacy of the control measures.

The data presented here suggest heterogeneity in resistance levels at a small geographical scale in the Yucatan state. A study done in the Caribbean found a wide variation in spatial heterogeneity of insecticide resistance on a much more local neighborhood scale [63] and similar results from an ongoing study in Merida is finding heterogeneity in resistance patterns within the city (Che Mendoza personal communication). This study had originally included analysis at the block level for each community, but egg collection and hatch rates limited the ability to pursue this.

These initial findings also suggest human movement and insecticide pressure could play role in the level of insecticide resistance in the towns surrounding Merida. San Lorenzo and Progreso showed the lowest mortality rates to deltamethrin (62.7% and 73.3%, respectively), as well as the highest frequency for both of the kdr mutations, with San Lorenzo showing the highest overall resistance for both phenotype and kdr frequency. Of the study towns, San Lorenzo was located closest to Merida (18km), and observations made during sampling suggest strong connectivity through consistent and frequent transportation between the two localities. Additionally, the household surveys identified regular use of these inter-city buses, particularly for daily or routine activities such as work or shopping. In contrast, Hunucma (35 km from Merida) showed some of the lowest deltamthrin mortality rates (87.6%; although still considered resistant by WHO) and the lowest frequency of both of the kdr mutations among the five towns. Furthermore, Hunucma reported the lowest use of daily motorized transit and most transportation was through short distance travel such as motorized taxi and walking,

These results suggest that proximity and connectivity to Merida, a city with high levels of insecticide resistance and high kdr frequencies, may be an associated risk factor for resistance. However, the dissimilar resistance profiles of Progreso and Hunucma, both equidistant from Merida (35km), indicate there are other factors that also play a role in how, and to what level, resistance emerges in a population. Progreso is an important port and is connected to Merida via a four-lane highway, and it is a common destination for vacationers from Merida due to its location on the coast. In contrast, Hunucma is a small rural town connected to Merida by small, 2-lane road. These preliminary findings show that the type and regularity of movement between towns may potentially contribute

to insecticide resistance levels. Finally, insecticide application pressure within communities is known to be a primary cause for resistance development [33]. All five towns studied met WHO criteria for resistance to deltamethrin. Although at varying degrees, each has histories of insecticide application by the Yucatan health authorities and by residents in their homes.

This study presents key findings regarding the heterogeneity of insecticide resistance, although these results are limited due to the cross sectional nature of the study design and the small number of insecticides and resistance mechanisms investigated. It is important to repeat this type of data collection in these communities to assess how insecticide use and other factors can influence resistance patterns while explaining how resistance can change in space and time. Additionally, it would be beneficial to expand the number of communities where data are collected and do so on a much more focal level, such as the block level.

As the world becomes more globalized and cities continue to expand, blurring the boundaries between urban centers and rural communities, the environments suitable for supporting *Ae. aegypti* will increase. This will likely lead to increased insecticide use in vector control, causing selection pressure and resulting in resistant mosquitoes. Additionally, the role human movement might have on inadvertently introducing resistance genes into populations should be explored further, particularly in places such as the Yucatan where there is regular movement and connectivity between the outlying communities (where selection pressure is light) and Merida (where selection pressure is high and resistance genes are present at a high frequency). For these reasons, understanding insecticide resistance mechanisms and how resistance enters into and

moves through a population will become increasingly important for dengue prevention and control programs.

Until these processes and their interactions are better understood vector control authorities will need to continue using the tools they have to control vector populations, in the midst of the growing threat of insecticide resistance. The limited number of suitable insecticides and logistical constraints makes focal vector control particularly challenging, but this is a challenge that must be considered given the small-scale heterogeneity of insecticide resistance. The prevention of resistance development and the management of resistance that has already been detected should be incorporated into vector control strategies. The use of multiple insecticides at a time through rotations or mixtures has been suggested, and evidence suggests that this could be an effective way to mitigate or stall the emergence of resistance [64, 65], at least in the short term. The ability to do this on a local or community scale though would be challenging and complex, requiring local authorities and technicians who can regularly monitor resistance within the population and have the power and ability to effectively implement a proper control strategy. This study describes several of the key components influencing insecticide resistance levels between towns in a highly dengue endemic area of Mexico. It is our hope these findings can help provide a basis for an evidence-based vector control strategy for these towns, and ultimately assist in preventing dengue transmission.

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Tables

Town	Population	Distance from Merida (km)
San Lorenzo de Uman	39,611	18
Acanceh	10,968	31
Progreso	37,369	35
Hunucma	24,910	35
Conkal	7,173	15

Table 1. Study site characteristics by population and distance from Merida

Insecticide	**Insecticide Concentration for *Aedes* species (µg/bottle)**	**Diagnostic Time (minutes)**
Bendiocarb	12.5	30
Deltamethrin	10	30
Chlorpyrifos	50	30

Table 2. CDC Bottle Bioassay diagnostics for tested insecticides. Mortality is measured at the diagnostic time after exposure to the diagnostic dose of the insecticide.

Town	House Index (%)	Container Index (%)	Breteau Index	Adult Index	Average number of people per house (SD)	Average number of adult female *Ae. aegypti* per person
San Lorenzo	16.33	9.04	32.65	3.24	4.1 (1.5)	0.81
Acanceh	24.00	13.95	72	2.13	4.7 (1.9)	0.45
Progreso	10.00	3.80	14	1.12	3.7 (1.9)	0.30
Hunucma	32.00	13.04	90	2.44	4.8 (2.0)	0.55
Conkal	54.00	27.72	102	2.23	4.2 (2.2)	0.53

Table 3. Mosquito infestation indices by study site; **House Index**: % of houses with larvae and/or pupae, **Container Index**: % of water holding container infested with larvae or pupae, **Breteau Index:** number of positive containers per 100 houses inspected, **Adult Index:** Average number of adult female *Ae. aegypti* per house.

		Sella's Stages % (n)					
	Total	**1**	**2**	**3**	**4**	**5**	**6**
San Lorenzo	162	58.0 (94)	27.2 (44)	0.6 (1)	1.2 (2)	0.0 (0)	12.9 (21)
Acanceh	108	28.7 (31)	6.5 (7)	17.6 (19)	15.7 (17)	19.4 (21)	12.0 (13)
Progreso	62	40.3(25)	0.0 (0)	9.7 (6)	17.7 (11)	25.8 (16)	6.5 (4)
Hunucma	123	25.2 (31)	19.5 (24)	8.1 (10)	13.8 (17)	20.3 (25)	12.2 (15)
Conkal	107	42.9(46)	14.9 (16)	10.3 (11)	14.9 (16)	11.2 (12)	5.6 (6)

Table 4. The abdomens of adult mosquitoes captured during indoor aspirator collections scored according to the Sella scale (1=unfed, 2=full bloodmeal, 3-5=partial bloodmeal, and 6=gravid)

		V1016I							F1534C				Double homozygotes	
Town	**Deltamethrin Phenotype**	**n**	**n***	**V/V**	**V/I**	**I/I**	**Freq. I**	**P***	**F/F**	**F/C**	**C/C**	**Freq. C**	**P***	**V/V & F/F**	**I/I & C/C**
San Lorenzo	susceptible	52	52	0	38	14	0.63		0	8	44	0.92		0	13
	resistant	31	31	1	4	26	0.9		1	0	30	0.97		1	26
	Total	**83**	**83**	**1**	**42**	**40**	**0.7349**	**<.0001**	**1**	**8**	**74**	**0.9398**	**0.012**	**1**	**39**
Acanceh	susceptible	72	72	19	45	8	0.42		7	40	25	0.63		7	8
	resistant	11	10	0	0	10	1.0		0	0	10	1.0		0	10
	Total	**83**	**82**	**19**	**45**	**18**	**0.4939**	**<.0001**	**7**	**40**	**35**	**0.6707**	**<.0001**	**7**	**18**
Progreso	susceptible	66	66	10	27	29	0.64		0	8	58	0.94		0	29
	resistant	24	24	0	7	17	0.85		0	0	24	1.0		0	17
	Total	**90**	**90**	**10**	**34**	**46**	**0.7**	**0.0021**	**0**	**8**	**82**	**0.9556**	**0.074**	**0**	**46**
Hunucma	susceptible	78	77	18	54	5	0.42		8	56	13	0.53		8	5
	resistant	11	11	0	2	9	0.91		0	0	11	1.0		0	9
	Total	**89**	**88**	**18**	**56**	**14**	**0.4773**	**<.0001**	**8**	**56**	**24**	**0.5909**	**<.0001**	**8**	**14**
Conkal	susceptible	74	70	8	45	17	0.56		7	39	24	0.62		4	15
	resistant	10	9	0	1	8	0.94		0	1	8	0.93		0	8
	Total	**84**	**79**	**8**	**46**	**25**	**0.6076**	**<.0001**	**7**	**40**	**32**	**0.6582**	**0.051**	**4**	**23**

Table 5. Summary of data relating deltamethrin resistance phenotype to kdr genotype per town. n*=number of individuals tested for kdr genotype from each community. V/V and F/F are SS (homozygous susceptible). V/I and F/C are SR (heterozygote) and I/I and C/C are RR (homozygous resistant).

Town	Allele	n	Frequency	95% Confidence Limits
San Lorenzo	I	82	0.735	±0.095
	C	82	0.939	±0.0512
	Total tested	83		
Acanceh	I	63	0.494	±0.1076
	C	75	0.671	±0.1011
	Total tested	83		
Progreso	I	80	0.7	±0.0947
	C	90	0.956	±0.0426
	Total tested	90		
Hunumca	I	70	0.477	±0.1038
	C	80	0.585	±0.1024
	Total tested	89		
Conkal	I	71	0.595	±0.105
	C	72	0.633	±0.1031
	Total tested	89		
Total	I	366	0.603	±0.0467
	C	399	0.759	±0.0408
	Total tested	422		

Table 6. The frequency of 1016I (I) and 1534C (C) at each town and the confidence interval around that frequency

Town	Table 7a.Times per Week Using Motorized Transit					
	n	Never	1-2	3-5	Daily	Monthly
San Lorenzo	49	0.10 (5)	0.14 (7)	0.08 (4)	0.65 (32)	0.02 (1)
Acanceh	50	0.1 (5)	0.24 (12)	0.08 (4)	0.54 (27)	0.04 (2)
Progreso	50	0.2 (10)	0.24 (12)	0.08 (4)	0.48 (24)	0.0 (0)
Hunucma	50	0.18 (9)	0.28 (14)	0.12 (6)	0.4 (20)	0.0 (0)
Conkal	50	0.08 (4)	0.24 (12)	0.24 (12)	0.44 (22)	0.0 (0)

	Table 7b.Most Common Type of Transit					
	n	Car	Bus/Combi	Moto-Taxi	Bicycle	Walk
San Lorenzo	49	0.12 (6)	0.61 (30)	0.22 (11)	0.06 (3)	0.0 0
Acanceh	50	0.08 (4)	0.14 (7)	0.52 (26)	0.2 (10)	0.2 (10)
Progreso	50	0.34 (17)	0.36 (18)	0.12 (6)	0.04 (2)	0.26 (13)
Hunucma	50	0.12 (6)	0.1 (5)	0.28 (14)	0.28 (14)	0.18 (9)
Conkal	50	0.26 (13)	0.2 (10)	0.24 (12)	0.2 (10)	0.08 (4)

Table 7c. Reasons for Traveling Outside of Town					
	n	Shopping	Work	Visiting Friends/Family	Other
San Lorenzo	49	0.31 (15)	0.37 (18)	0.2 (10)	0.22 (11)
Acanceh	50	0.48 (24)	0.28 (14)	0.04 (2)	0.46 (23)
Progreso	50	0.38 (19)	0.08 (4)	0.24 (12)	0.42 (21)
Hunucma	50	0.28 (14)	0.24 (12)	0.14 (7)	0.36 (18)
Conkal	50	0.34 (17)	0.3 (15)	0.06 (3)	0.4 (20)

Tables 7a-c. Household survey responses regarding the use of motorized transportation

Town	Reported dengue cases, n	Houses treated with propoxur, n	Houses treated with deltamethrin, n	Houses with no treatment, n
San Lorenzo	52	37	13	2
Acanceh	19	10	9	0
Progreso	88	65	16	7
Hunucma	49	32	16	1
Conkal	8	7	251	0

Table 8. Application of insecticides by the state vector control program in the homes of reported dengue cases during epidemiological weeks 1-35, 2013

Figures

Figure 1. Location of Merida, Yucatan, Mexico and the five study towns

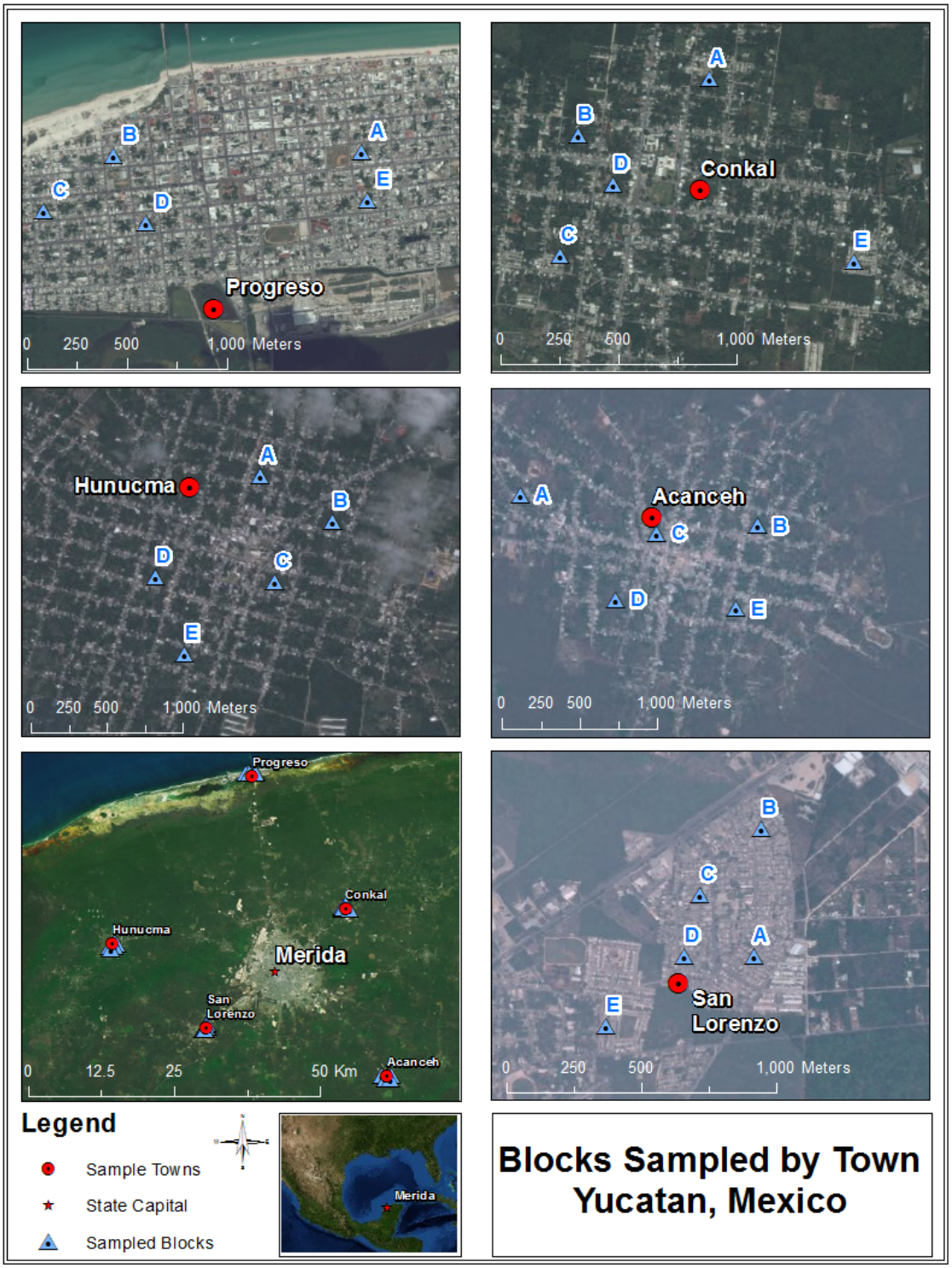


Figure 2. Location of the blocks that were sampled in each town

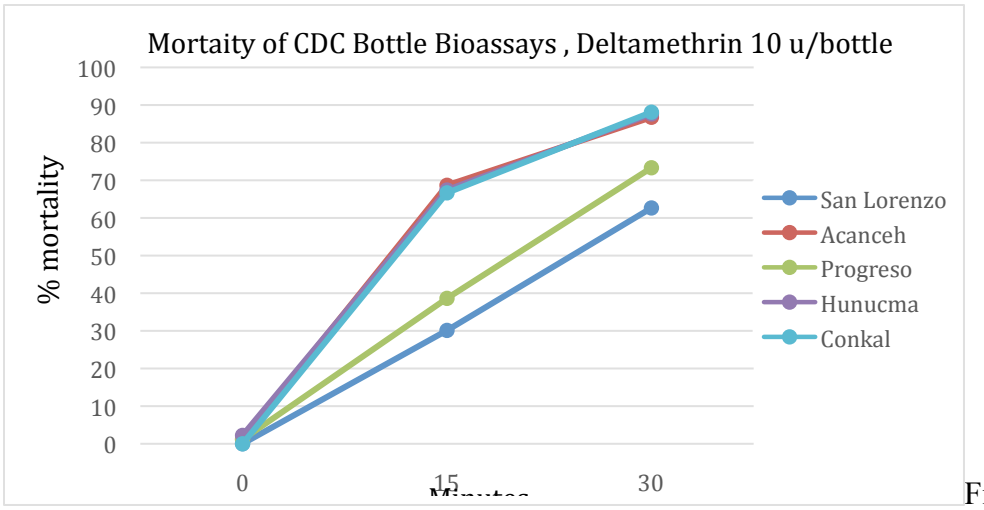


Figure 3a.

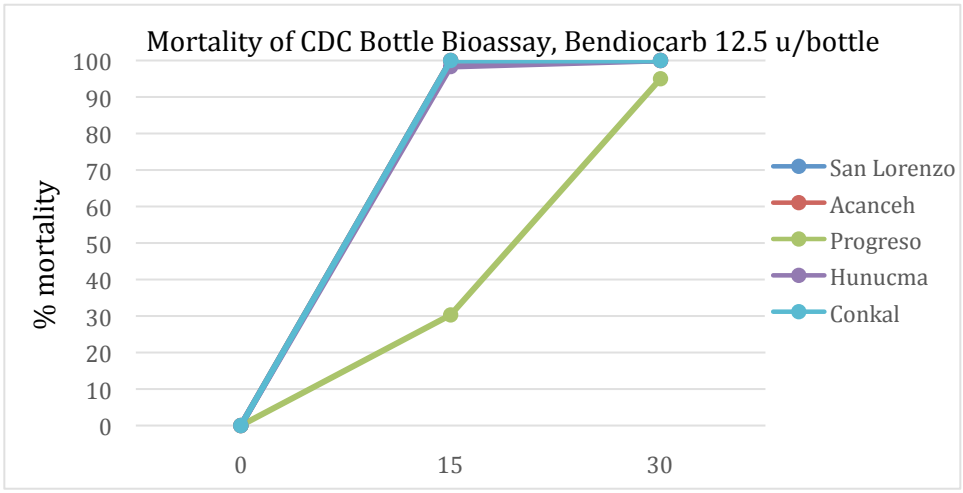


Figure 3b.

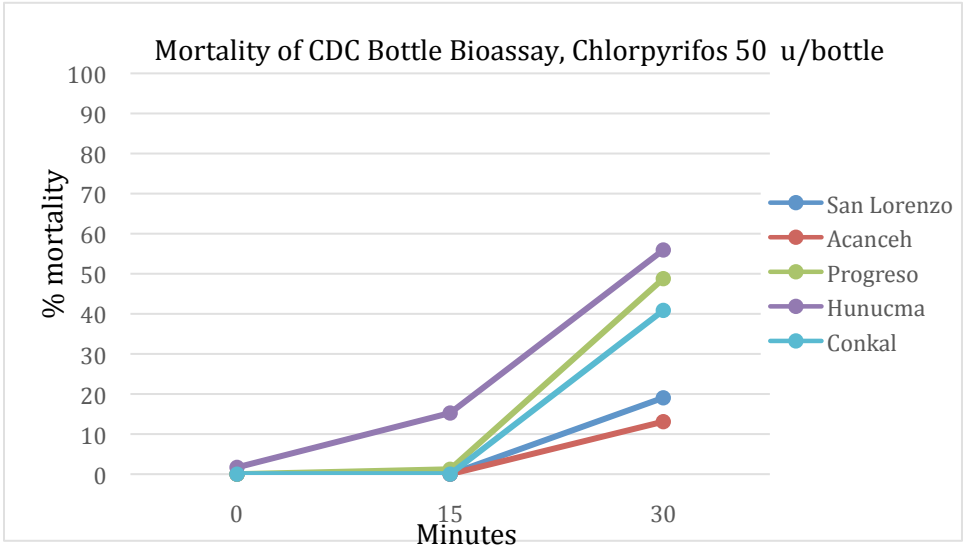


Figure 3c.

Figures 3a-3c: Knockdown times for female *Ae. aegypti* exposed to insecticide diagnostic doses after 30 minutes using the CDC Bottle Bioassay

Figure 4a

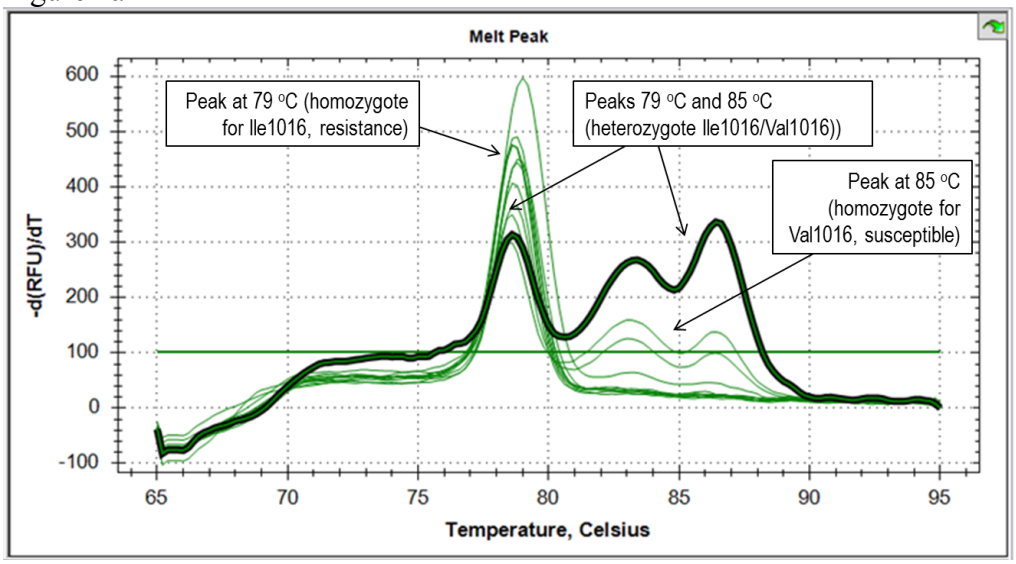
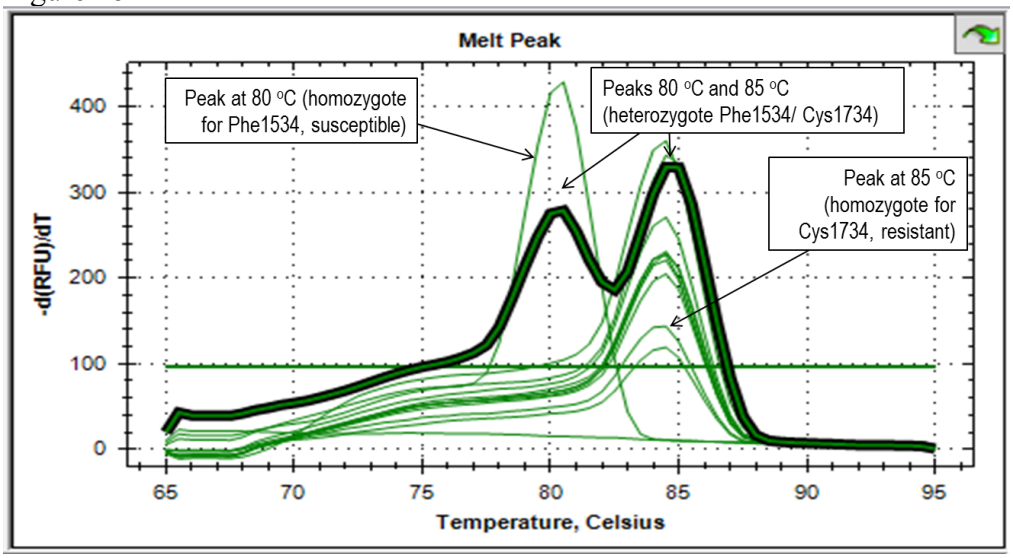
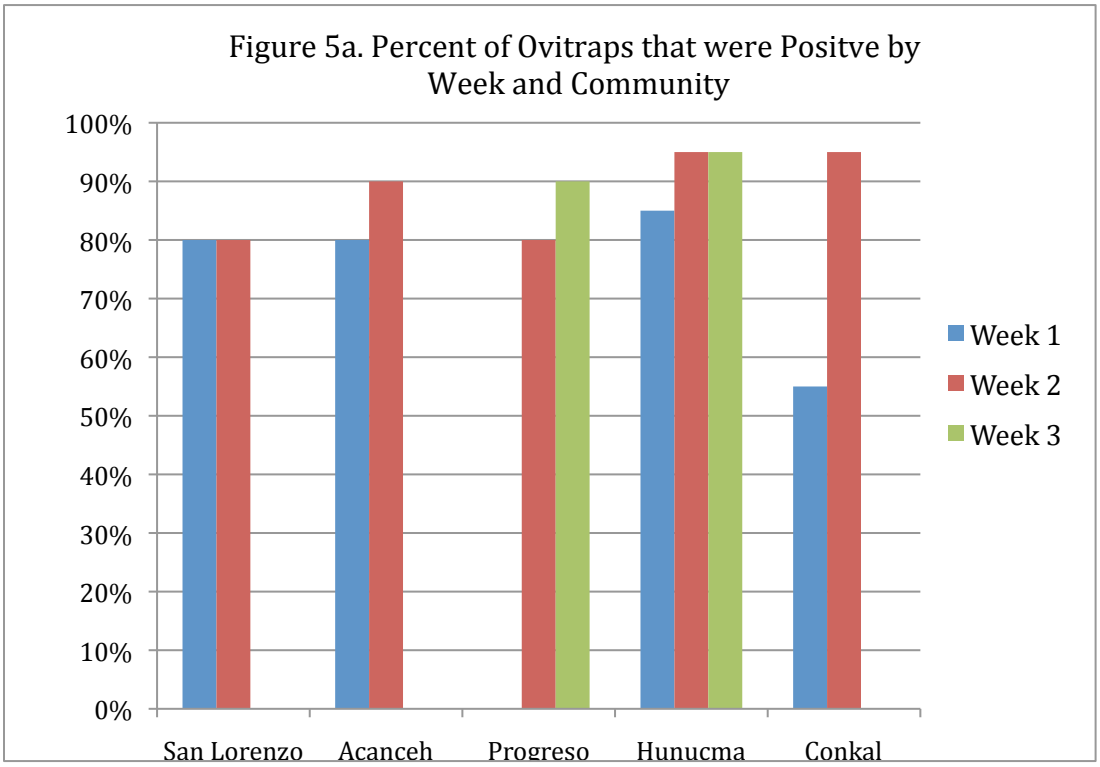
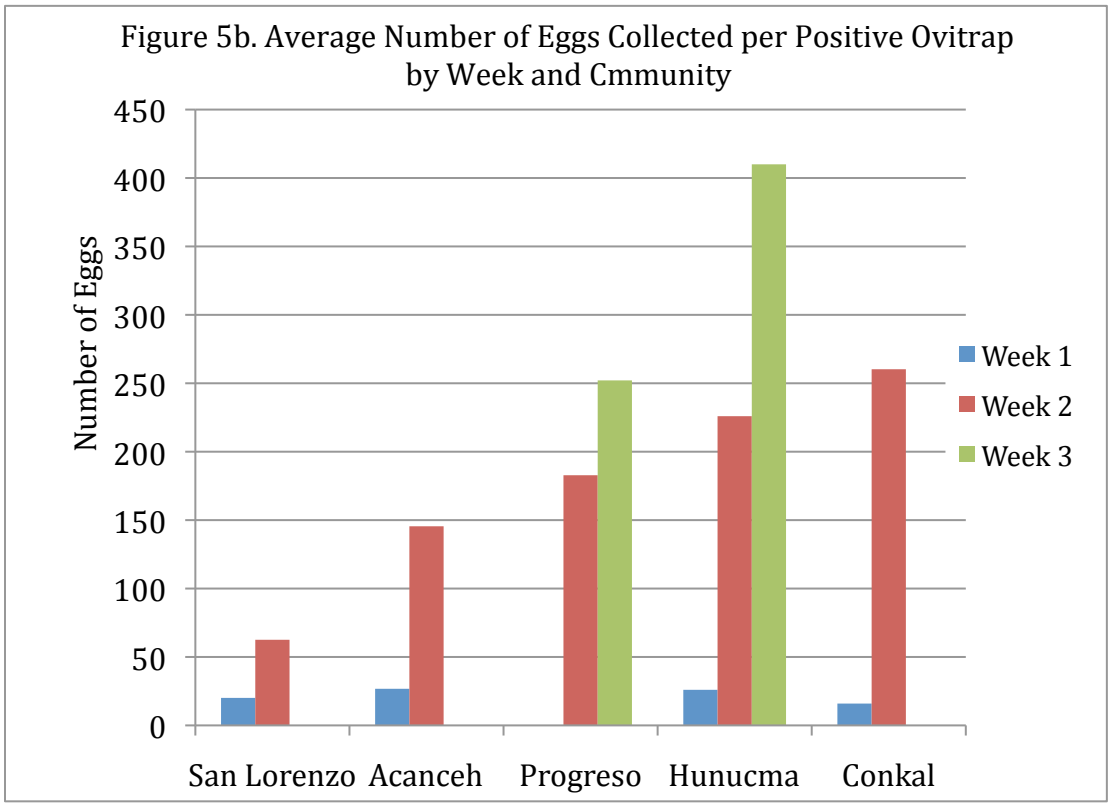


Figure 4b



Figures 4a. Melting curves for Iso1016/Iso1016, Val1016/Iso1016 and Val1016/Val1016.**4b.**Melting curves for Phe1534/Phe1534, Phe1534/Cys1534, and Cys1534/Cys1534





Figures 5. The percent of ovitraps positive for *Ae. aegypti* eggs by week in each town and the average number of eggs collected per positive ovitrap in each town per week