Permethrin Resistance Status and Associated Mechanisms in *Aedes albopictus* (Diptera: Culicidae) From Chiapas, Mexico

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Abstract

There are major public health concerns regarding the spread of mosquito-borne diseases such as dengue, Zika, and chikungunya, which are mainly controlled by using insecticides against the vectors, *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse). Pyrethroids are the primary class of insecticides used for vector control, due to their rapid knockdown effect and low toxicity to vertebrates. Unfortunately, continued use of pyrethroids has led to widespread insecticide resistance in *Ae. aegypti*; however, we lack information for *Ae. albopictus*—a sympatric species in Chiapas since 2002. In this study, we evaluated the permethrin resistance status of *Ae. albopictus* collected from Mexico and Texas. We also selected for permethrin resistance in the laboratory and investigated the potential mechanisms conferring resistance in this species. Knockdown resistance mutations, specifically F1534C, in the voltage-gated sodium channel gene, and increased activity of detoxifying enzymes were evaluated. Low levels of permethrin resistance (<2.4-fold) were observed in our field populations of *Ae. albopictus* and the F1534C mutation was not detected in any of the sites. Low levels of resistance were also observed in the artificially selected strain. There was significantly higher cytochrome P450 activity in our permethrin-selected and nonselected strains from Mexico compared to the control strain. Our results suggest the *Ae. albopictus* sampled from 2016 are mostly susceptible to pyrethroids. These results contrast with the high levels of permethrin resistance (>58-fold) found in *Ae. aegypti* from the same sites in Mexico. This research indicates the importance of continued monitoring of *Ae. albopictus* populations to prevent resistance from developing in the future.

Key words: permethrin, resistance, mosquitoes

In Mexico, *Aedes aegypti* (Linnaeus, Diptera: Culicidae) has historically been the major vector of arboviral diseases including Zika, dengue, chikungunya, and yellow fever. Unfortunately, an invasive species, *Ae. albopictus* (Skuse), was first reported in 1993 in Mexico and since then has gradually dispersed to 12 states of the country (Pech-May et al. 2016). Although *Ae. albopictus* is still considered a secondary vector of arboviral diseases in Mexico, the potential impact that *Ae. albopictus* can have on public health cannot be neglected. For example, *Ae. albopictus* is considered the primary vector of dengue in certain regions (Gratz 2004) and has also been implicated as the primary vector during several dengue epidemics (Gilbertson 1945, Effler et al. 2005). Additionally, invasive

Ae. albopictus was found to be the vector responsible for the chikungunya epidemic of 2005–2006 on Reunion island (Reiter et al. 2006) due to mutations in the envelope glycoprotein genes (E1 and E2) that enhanced the ability of the virus to be disseminated and transmitted by *Ae. albopictus* (Tsetsarkin et al. 2007, Tsetsarkin and Weaver 2011).

In Tapachula, Chiapas—our study site—*Ae. albopictus* was first reported in 2002 (Casas-Martínez and Torres-Estrada 2003) and has continued to spread throughout the state since then. Currently, *Ae. aegypti* and *Ae. albopictus* are sympatric species with overlapping breeding sites in the major cities and rural regions of Chiapas. Vector control campaigns in Mexico direct surveillance and control efforts

against *Ae. aegypti*, which feed almost exclusively on humans and typically rest indoors. However, methods of control also might be affecting *Ae. albopictus*, which usually exhibit exophagic behavior and feed on humans and animals opportunistically (Paupy et al. 2009) but also have the capability to exhibit anthropophilic behaviors similar to *Ae. aegypti* in specific contexts (Ponlawat and Harrington 2005, Delatte et al. 2010).

Methods of mosquito control in Mexico include the physical removal of breeding sites, use of the larvicide Abate, *Bacillus thuringiensis* var. *israelensis* and Spinosad, and outdoor spraying of ultra-low volume (ULV) insecticides targeting infected adult mosquitoes during dengue outbreaks. One of the most suitable classes of insecticides formulated for ULV sprayings is pyrethroids. These insecticides became popular because they have a rapid knockdown effect and low toxicity to humans and other mammals (WHO/CDC/WHOPES/GCDPP 2005).

Exclusive and prolonged use of pyrethroids in vector control programs resulted in selection of mechanisms that confer resistance in *Ae. aegypti* populations in Mexico (Aponte et al. 2013, Flores et al. 2013). However, information about *Ae. albopictus* susceptibility to insecticides is scarce in Mexico. Since both species have been sympatric in Chiapas during the last 18 yr, we would expect both species to have developed mechanisms of resistance to pyrethroids. Pyrethroid resistance has been reported in *Ae. albopictus* from several countries, including Sri Lanka (Karunaratne et al. 2013), Pakistan (Arslan et al. 2016), and China (Chen et al. 2016, Xu et al. 2016).

There are two main mechanisms that confer resistance in insects and reduce the effectiveness of these insecticides. One is caused by target site insensitivity, which occurs when one or more point mutations in the voltage-gated sodium channel gene (*vssc*) result in knockdown resistance (*kdr*) to the insecticide (Yu 2015). Several *kdr* mutations associated with resistance have been identified in *Ae. albopictus* throughout multiple countries, including I1011M/V (McAllister et al. 2012), V1016G (Kasai et al. 2019, Zhou et al. 2019), I1532T (Xu et al. 2016, Gao et al. 2018, Zhou et al. 2019), and F1534L/S (Marcombe et al. 2014, Chen et al. 2016, Xu et al. 2016, Gao et al. 2018, Li et al. 2018, Kasai et al. 2019, Zhou et al. 2019). Additionally, F1534C was found in Singapore (Kasai et al. 2011), Greece (Xu et al. 2016), China (Chen et al. 2016, Gao et al. 2018), Brazil (Aguirre-Obando et al. 2017), and Vietnam (Kasai et al. 2019). However, none of the *kdr* mutations have been functionally confirmed to confer pyrethroid resistance in *Ae. albopictus*.

The second major mechanism is known as metabolic resistance, which occurs when the amount or activity of detoxification enzymes are enhanced or modified, which then metabolize the insecticides and prevent them from attaching to the target site (Brogdon and McAllister 1998). Three major groups of enzymes have been implicated in metabolic resistance to pyrethroids: cytochrome P450 monooxygenases (P450s; a.k.a. mixed function oxidases), glutathione *S*-transferases (GSTs), and esterases (Hemingway et al. 2004, Yu 2015).

The goal of this study was to determine the baseline permethrin susceptibility levels of 11 field populations of *Ae. albopictus* collected from Southern Mexico in and around the city of Tapachula, Chiapas. We also tested *Ae. albopictus* collected from Southern Texas in order to compare susceptibility levels between different geographical locations. Additionally, we performed artificial selection with permethrin over several generations in *Ae. albopictus* collected from Chiapas and investigated the target site insensitivity (*kdr* mutations) and detoxification enzyme activity as possible mechanisms for resistance in the *Ae. albopictus* selected strain. Since vector control strategies targeting *Ae. aegypti* have resulted in high levels of resistance to permethrin

and other pyrethroid insecticides in Mexico (Flores et al. 2009, 2013; Aponte et al. 2013; Kuri-Morales et al. 2018; Lopez-Monroy et al. 2018), we hypothesized that these strategies have also affected the status of susceptibility in *Ae. albopictus* from Chiapas.

Materials and Methods

Mosquito Collections

Mosquito collections were performed by the Centro Regional de Investigación en Salud Pública (CRISP) in the state of Chiapas, Mexico in September 2016. Figure 1 shows the geographical position of the collection sites. Four sites were sampled within the city of Tapachula (Los Llanes, Col. 5 de Febrero, San Agustín, and El Porvenir). At these sites, larvae and pupae were collected from a wide variety of containers associated with households (tires, cups, buckets, outdoor sinks). The remaining seven sites consisted of rural towns including Puerto Madero, Huehuetán, Huixtla, Escuintla, Motozintla, Mapastepec, and Pijijiapan. At these sites, larvae and pupae were collected from flowerpots and vases within cemeteries. The collected larvae and pupae (F_0) were brought back to the insectary at CRISP and were identified and sorted by species upon emergence. Adults were blood-fed on rabbits and F_1 eggs were collected for each site. Additionally, *Ae. albopictus* eggs from Weslaco, TX were collected by the Dr. Gabriel Hamer laboratory (Texas A&M University) funded through the Western Gulf Center of Excellence for Vector-Borne Diseases. Eggs (F_1) from three sites in Weslaco: West Mile 10 (WM10), Weslaco City Cemetery (WCC), and Estero Llano Grande (ELG) via ovitraps between May and June of 2018.

The *Ae. albopictus* control strain, ATM-NJ95, was obtained from Biodefense and Emerging Infections Research Resources Repository (BEI Resources) as a confirmed insecticide-susceptible population (Marcombe et al. 2014). This reference strain was originally established from larvae collected near Keyport, NJ in 1995, shortly after *Ae. albopictus* was first detected in the state (Crans et al. 1996).

Eggs (F_1) from Mexico and Texas, and ATM-NJ95 were shipped to CSU (Fort Collins, CO) with the CDC import permit PHS-2017-08-33, and were hatched and maintained in the laboratory under insectary conditions (60–80% RH, 28–30°C). Eggs were hatched in 4-liter clear plastic tubs with approximately 2 liters of autoclaved tap water and larvae were fed a 10% liver powder solution (MP Biomedicals LLC, Irvine, CA). On the first day that the eggs were hatched, 1 ml of 10% liver powder solution was added to each tub. Three days after hatching, larvae were divided into multiple tubs, each containing about 200–300 larvae, and were fed 2 ml of 10% liver powder solution daily in order to provide similar rearing conditions and to obtain relatively equal body size among all mosquito strains. Pupae were removed from larval tubs and placed in BugDorm-1 cages (Mega View Science Co., Taichung, Taiwan). Adult mosquitoes were given raisins *ad libitum*.

Bottle Bioassays

Bottle bioassays were adapted from the CDC bottle bioassay protocol (CDC 2013) to determine the lethal concentration that kills 50% (LC_{50}) of the population. The active ingredient consisted of a mix of *cis* and *trans* isomers of permethrin (PESTANAL, Sigma-Aldrich, St. Louis, MO). To prepare bottles, 1 ml of acetone was pipetted into each bottle, and then the appropriate amount of working permethrin solution was pipetted into each bottle to achieve the desired concentrations (0, 0.5, 1.0, 1.5, 2.0, and 5.0 µg per bottle). Each concentration was tested in triplicate for every mosquito strain,

Fig. 1. Map of *Ae. albopictus* collection sites in and around Tapachula, Chiapas, Mexico.

using three different bottles (of the same concentration) for each replicate. Bottles were reused a maximum of three times.

Bottle bioassays were conducted with the F_3 generation. Adult mosquitoes 3–5 d postemergence were aspirated into the bottles using a mouth aspirator. We aimed to have 15 male and 15 female mosquitoes per bottle. The mosquitoes were left in the bottles for 60 min and then transferred to recovery cups, provided cotton balls soaked in a 10% sucrose solution and were left in insectary conditions. After 24 h, the number of dead mosquitoes was recorded.

Statistical Analysis of Bottle Bioassays

The LC_{50} and the 95% highest density intervals (95% HDIs) were calculated for every strain by using a binomial logistic regression Bayesian analysis on SAS software. Resistance ratios (RRs) and intervals were calculated by dividing the LC_{50} and 95% HDIs of the test strain with the LC_{50} and 95% HDIs of the control strain (ATM-NJ95). We considered RRs to be significantly different when the RR 95% HDIs did not overlap between strains (Kruschke 2014). We used the criteria proposed by Mazzari and Georgiou (1995) to classify RRs as high (>10-fold), moderate (between 5- and 10-fold), and low (<5-fold) levels. Since we could not test all the mosquito strains at one given time, we had to re-assay ATM-NJ95 every time we assayed a set of test strains together. Refer to [Supp Table 1](#) (online only).

Artificial Permethrin Selection

We created a laboratory permethrin-selected strain, which from this point on will be referred to as the La Macha strain. The 11 colonies from Chiapas, Mexico were tested via bottle assays at the F_3 generation, and the survivors from the assays were kept and allowed to interbreed. Additionally, we also used this same pool of mosquitoes to create a nonselected strain known as La Delicada. The mosquitoes of the La Delicada group were exposed to permethrin at the F_3

generation, prior to pooling the collection sites. However, the major difference between the two groups was that La Delicada had no further exposure to permethrin from that point on, while La Macha was repeatedly selected with permethrin at the F_3 , F_7 , F_9 , and F_{11} generations. Selection was carried out using the same bottle assay procedures and statistical analyses as described in the previous sections, which means that the mosquitoes were pressured with a range of concentrations, rather than a single concentration.

Screening the F1534C mutation in the *vgsc* of *Aedes albopictus*

For this objective, DNA was extracted from 25 females and 25 males of the F_1 generation from each of the 11 collection sites using Pat Roman's grinding buffer and 8 M potassium acetate (Black and DuTeau 1997). DNA pellets were purified with ethanol and resuspended in 180 μ l of TE buffer (1 M Tris-HCl, 0.5 M EDTA, pH 8.0).

We used previously published primers to amplify the genomic region flanking the F1534 residue in the *vgsc* of *Ae. albopictus* (Kasai et al. 2011). PCR reactions were prepared in low 96-well clear Multiplate PCR Plates (Bio-Rad Laboratories, Hercules, CA) by mixing 1 μ l of DNA with 12.5 μ l GoTaq Green Master Mix (Promega, Madison, WI), 11.4 μ l ddH₂O, and 25 μ M of each forward (aegSCF7) and reverse primers (aegSCR8) (see [Table 1](#)). The final reaction (25 μ l) was placed in a MyCycler thermal cycler (Bio-Rad Laboratories) following the conditions: 95°C for 5 min, 33 cycles of 95°C for 30 s, 52°C for 30 sec, 72°C for 1 min, followed by a final extension at 72°C for 5 min. PCR products were checked for quality in a 2.0% agarose gel and purified using the MiniElute Qiagen kit (Hilden, Germany). PCR products were processed by Genewiz Sanger Sequencing (South Plainfield, NJ) using the sequencing primers IIIS6short+ and IIIS6short– shown in [Table 1](#).

Table 1. List of primer sequences used for Sanger Sequencing and AS-PCR protocols to screen for mutations at the 1534 site of the Domain III Segment 6 region of the *vgsc* for *Ae. albopictus*

Primer function	Primer name	Primer sequence	Product size (bp)
Amplification	aegSCF7	5'-GAGAACTCGCCGATGAACCTT-3'	413–429
	aegSCR8	5'-TAGCTTTCAGCGGCTTCTTC-3'	
Sequencing	IIIS6short+	5'-AACGATCGTTTCTCTTGA-3'	172
	IIIS6short-	5'-CCGGCTTCTTCTTCTGC-3'	
AS-PCR	C1534_albo	5'- <u>GCGGGCAGGGCGGGCGGGGGCGGGG</u> CCTCTACTTYGTGTTCTTCATCATG-3'	113
	F1534_albo	5'- <u>GCGGGCTCTACTTYGTGTTCTTCATCAT</u> ATT-3'	93
	1534rev_albo	5'-TCTGCTCGTTGAAGTTGTCGAT-3'	

For the AS-PCR primers, the gray-highlighted sequences are the long and short GC tails assigned to the forward primers for the Cysteine mutation sequence and the Phenylalanine wild-type sequence, respectively. Underlined letters indicate intentionally mismatched nucleotides to help improve assay specificity. The allele-specific nucleotides at the –3' ends are shown in bold (G codes for Cysteine, T codes for Phenylalanine).

Once we obtained a consensus sequence of the exons 28–30 in the *vgsc* in mosquitoes from Chiapas ($n = 14$), we proceeded to design an allele-specific PCR (AS-PCR) system that allowed us to track specifically the F1534C substitution in genomic DNA of *Ae. albopictus*. Allele-specific primers were designed following Okimoto and Dodgson (1996), Saavedra-Rodriguez et al. (2007), and Yanola et al. (2011). Briefly, the system consisted of two forward primers that contain the allele-specific nucleotide at the 3'-end and a common reverse primer. Long (26 mer) and a short (6 mer) GC tails were attached to the 5'-end of the AS-PCR primers to distinguish the amplified products based on size. The long tail was assigned to the Cysteine substitution (C1534_albo primer) and the short tail was assigned to the wild-type Phenylalanine (F1534_albo primer) (Table 1). Primers were resuspended to 50 pmol/μl in ddH₂O.

AS-PCR samples were prepared in 96-well white Multiplate PCR Plates (Bio-Rad Laboratories) by mixing 1 μl of DNA, 9.53 μl of ddH₂O, 10.0 μl of iQ SYBR Green Supermix (Bio-Rad Laboratories), 0.066 μl of C1534_albo primer, 0.20 μl of F1534_albo, and 0.20 μl of 1534 rev_albo primer. Wells were covered with Optical Flat 8-Cap Strips (Bio-Rad Laboratories), centrifuged for 2 min and placed in the CFX Connect Real Time System thermal cycler (Bio-Rad Laboratories). The plates were run under the following thermocycler conditions: 95°C for 3 min, 39 cycles of 95°C for 10 s, 57°C for 10 s, 72°C for 30 s, followed by 95°C for 10 s. Melting curve conditions were 65°C to 95°C in 0.5°C increments every 5 s.

The AS-PCR assay was optimized using a synthetic sequence containing the F1534C substitution (C1534+control). The 222-bp sequence flanking the F1534C substitution was designed using the consensus alignment of our *Ae. albopictus* sequences and synthesized in plasmids (IDT, Coralville, IA). Supp Table 2 (online only) shows the sequence and highlights the T to G introduced at the second codon position at the 1534 residue. The C1534+control was suspended in TE to a final 15 ng/μl concentration. We validated the specificity of the AS-PCR using DNA of sequenced wild-type individuals (F1534+control), the C1534+control (0.0015 ng/μl), and mixtures of the F1534+control and C1534+control to test for heterozygote (F1534/C1534) limits of detection. The three-control PCR reactions were run on 3.5% agarose gel at 80 Volts for 60 min to confirm appropriate amplicon lengths.

We obtained the genotypes at the F1534C locus in 50 F₁ mosquitoes sampled from each of the 11 collection sites in Chiapas, Mexico, the F₁₁ of La Macha (survivors from bottle assays), the F₁₁ La Delicada, and ATMNJ95 (25 males and 25 females per strain).

Biochemical Assays

To test the possible enhanced metabolism of the laboratory-selected and nonselected strains, we measured the enzymatic activity of

individual mosquitoes following the biochemical assay protocol from Valle et al. (2006). The enzymatic activity for multiple function oxidases (MFO), acetylcholinesterases (AChE), alpha-esterases (α-EST), beta-esterases (β-EST), p-nitrophenyl acetate esterases (PNPA), glutathione S-transferases (GSTs), and proteins (PTN) was calculated for 40 nonblood-fed females (4 d postemergence) from each of the following strains of *Ae. albopictus*: the permethrin-selected La Macha (F₁₁), nonselected La Delicada (F₁₁), and the susceptible control strain, ATM-NJ95. Enzyme absorbance values of each mosquito were measured using Bio-Rad Benchmark Plus microplate spectrophotometer and Microplate Manager 5.2.1 software.

Statistical Analysis of Biochemical Assays

Enzyme activity was calculated from the measured absorbance values following the calculations from Valle et al. (2006). The amount of total protein in each mosquito was calculated using a standard curve obtained from bovine serum albumin (BSA). The absorbance values of the MFO, α-EST, and β-EST were converted to appropriate enzymatic activity measurements by using standard absorbance curves with known quantities of cytochrome C, α-naphthol, and β-naphthol, respectively. The enzyme-specific absorbance was normalized by the amount of total protein calculated for each individual mosquito. We used R 3.6.0 software to calculate one-way analysis of variance (ANOVA) and pairwise *t*-tests (with Bonferroni adjustment) and to graph boxplots to compare differences in mean enzyme activity between strains. The Bonferroni-corrected *P*-value was 0.017 [0.05 (original *P*-value)/3 (# of comparisons)].

Results

Insecticide Susceptibility of Field *Aedes albopictus*

Overall, the *Ae. albopictus* in this study had low levels of permethrin resistance relative to the susceptible control strain. Out of the field sites from Mexico, the mosquitoes from Col. 5 de Febrero had the highest RR, 2.00 (1.76–2.40), while mosquitoes from Puerto Madero had the lowest RR, 1.18 (0.93–1.44) (Fig. 2; Supp Table 1 [online only]). The RR of site Col. 5 de Febrero was significantly higher than the RRs of Los Llanes, San Agustín, Puerto Madero, Huixtla, and Escuintla. There were no distinct resistance patterns observed when comparing sites from within the city of Tapachula to rural/suburban sites outside of the city (Fig. 2).

The *Ae. albopictus* from Texas also had low levels of resistance relative to the control strain. Mosquitoes from WCC had the highest RR, 2.40 (2.09–3.11), while WM10 had the lowest RR, 1.37 (1.28–1.59). The RR of WM10 was significantly lower than the RRs of WCC and ELG (Fig. 2; Supp Table 1 [online only]). The most resistant strain from Texas, WCC, was significantly higher than all the

Fig. 2. Permethrin RRs in *Ae. albopictus* from Mexico and Texas. The three top sites were collected in Texas. Sites located in Tapachula City include Los Llanes, Col. 5 Febrero, San Agustin and El Porvenir. The remaining sites are from towns outside of Tapachula listed in descending order from closest to furthest from the city. RRs and intervals were calculated by dividing the LC₅₀ and 95% HDIs of the test strains by the LC₅₀ and 95% HDIs of the control strain (ATM-NJ95). RRs ≤ 1 would imply the test strain is equally susceptible or more susceptible to permethrin than the control strain. Nonoverlapping intervals suggest a significant difference between strains.

strains from Mexico except for Col. 5 de Febrero and Motozintla (Fig. 2; Supp Table 1 [online only]).

Artificial Permethrin Selection of *Aedes albopictus* From Mexico

The La Macha strain was selected with permethrin a total of five times at the F₃, F₅, F₇, F₉, and F₁₁ generations. The F₅ generation had a RR of 1.75 (1.68–1.86), the F₇ generation had a RR of 2.04 (1.71–2.75), the F₉ generation had a RR of 2.26 (1.99–3.15), and the F₁₁ generation had a RR of 1.28 (1.23–1.44) (Fig. 3; Supp Table 1 [online only]). The F₃, F₇, and F₉ generations all demonstrated an increasing trend in RRs over time, with the F₉ generation being significantly higher than the F₅ generation. However, the RR significantly dropped at the F₁₁ generation. The 95% HDI of the La Macha F₁₁ generation did not overlap with any of the other previously selected generations.

Genotyping the F1534C Locus in *Aedes albopictus*

The functional role of F1534C in the VGSC has not been demonstrated in *Ae. albopictus*; however, because of the high conservation between *Aedes* *vgsc* and the confirmed functional role of F1534C in *Ae. aegypti* *vgsc* in the presence of pyrethroids (Du et al. 2013), we focused our efforts to identify the F1534C in *Ae. albopictus* from Mexico. F1534C is the most common nonsynonymous mutation identified to date in *Ae. albopictus* worldwide, including the American continent. Figure 4 shows the partial genomic annotation of the consensus sequence spanning exons 28 and 29 of the *Ae. albopictus* *vgsc* gene. We obtained successful sequences from

Resistance Ratios of M.X. *Ae. albopictus* with and without Permethrin Selection

Fig. 3. RRs and intervals of the permethrin-selected (La Macha) and nonselected (La Delicada) are listed in descending order of generation number for each strain. RRs and intervals were calculated by dividing the LC₅₀ and 95% HDIs of the test strains with the LC₅₀ and 95% HDIs of the control strain (ATM-NJ95). RRs ≤ 1 would imply the test strain is equally susceptible or more susceptible to permethrin than the control strain. Nonoverlapping intervals suggest a significant difference between strains.

six ATMNJ95 females, three Motozintla F₁ females, and five La Macha F₁₁ females that survived the last bottle bioassay. In a consensus sequence of 278 bp, we identified seven polymorphisms and a 16-nucleotide insertion/deletion within intron 28–29. Additionally, seven synonymous mutations were identified in exon 29 (Fig. 4). The F1534C conferring mutation was not identified in any of the sequences, including individuals from the surviving La Macha F₁₁ females.

To screen a larger number of individuals, we designed an AS-PCR to identify the F1534C mutation in the *vgsc* of *Ae. albopictus*. Figure 5A shows the melting curve peaks of the Phenylalanine homozygote or wild-type genotype (F1534/F1534) with a single peak at 80°C and the Cysteine homozygote-resistant genotype (C1534/C1534) which had a single peak at 85°C. The heterozygote (F1534/C1534) presented peaks at both 80°C and 85°C. The size difference (20 bp) between AS-PCR amplicons was confirmed in a 3.5% agarose gel (Fig. 5B).

We screened for the presence of F1534C in genomic DNA of 700 *Ae. albopictus*. All mosquitoes collected in the 11 sites of Chiapas, Mexico ($n = 550$) were homozygous for the wild-type allele (F1534/F1534). If the mutation is present in the field, it might be at frequencies lower than 0.0018 (1 out of 550) and in heterozygous state.

Additionally, we screened for the F1534C in the *Ae. albopictus* strains subjected to permethrin selection (La Macha F₁₁), its sister strain that was not subjected to permethrin selection (La Delicada F₁₁) as well as the susceptible strain (ATM-NJ95). All mosquitoes were homozygous for the wild-type allele (F1534/F1534).

Aedes albopictus* VGSC (vector base =AAFL000723), partial exon 28-29*Exon 28 (E1,414)**

E N S P M N F D H V G K A Y L C L F Q V
GAGAACTCGCCGATGAACTTCGACCACGTGGGGAAGGCGTACCTGTGTCTGTTCCAGGTG
aegSCF7

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

intron 28-29 (67-83 bp) indel

tcgggatcttcgatcatcwcacatcagttcagccccraatcaatycgaytaacgatcgtttc
IIIS6short+

Exon 29

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

F1,534 (F1,474)

L Y F V F F I I F G S F F T L N L F I
 CTCCTATTYGTGTTCTTCATCATCTTCGGGTCGTTCTTCACCCTYAACTGTTTCATC
 5' [L-] CTCCTATTYGTGTTCTTCATCATCTTC-3' C1,534_albo
 5' [S-] CTCCTATTYGTGTTCTTCATCATCTTC-3' F1,534_albo

Forward AS-PCR primers

G V I I D N F N E Q K K K A G G S L E M
 GGTGTCATCATCGACAACCTCAACGAGCAGAAGAAGAAAGCCGGTGGCTCGCTGGAAATG
 3' -TAGCTGTTGAAGTTGCTCGTCT-5' 1534rev_albo
Reverse AS-PCR primer IIIS6short-

F M T E D Q K K Y Y N A M K K M G S K K
 TTCATGACGGAGGATCAGAAAAAGTACTACAACGCAATGAAAAAGATGGGCTCGAAGAAG
 3' -CTTCTTC

P L K A I
CCGCTGAAAGCTA
 GGCGACTTTCGAT-5'
aegSCR8

Fig. 4. Partial annotated sequence of exons 28 and 29 in *Ae. albopictus* voltage-gated sodium channel gene (*vgsc*). Primers used for Sanger Sequencing and AS-PCR genotyping protocols are also shown and labeled. The allele-specific nucleotides of the forward AS-PCR primers are highlighted in pink (G codes for Cysteine, T codes for Phenylalanine). Gray-highlighted nucleotides indicate a synonymous polymorphic site.

Biochemical Assays

Since we had originally anticipated the La Macha F₁₁ generation to have the highest RR, we expected to see higher enzyme activity in this strain relative to the La Delicada F₁₁ and the ATMNJ95 (susceptible reference strain). Table 2 shows the mean enzyme activity, standard errors, and pairwise comparisons between the means of the three strains.

Significant differences between La Delicada and La Macha occurred in the α -EST ($P = 0.0076$), β -EST ($P = 0.0012$), GST ($P = 0.0044$), and PNPA ($P = 2.00E-09$). Interestingly, mean activities were higher in La Delicada strain (Table 2), except for the activity of MFO (P450s). Notably, the two Mexico strains (La Macha and La Delicada) had equal levels of P450 activity ($P = 1$) and were both significantly higher than the ATMNJ95 strain ($P < 0.017$). Figure 6 shows the boxplot comparisons of P450 activity between the three strains.

Significant differences between ATMNJ95 and La Delicada occurred in AChE ($P = 0.0087$), P450 ($P = 4.00E-08$), and PNPA ($P = 0.002$) mean activities. In this comparison, the mean activity was significantly higher in La Delicada (Table 2). Additionally, between the ATMNJ95 and La Macha comparison, β -EST ($P = 0.0028$) and PNPA ($P = 0.004$) were significantly lower in La Macha.

Discussion

Low levels of permethrin resistance were observed in populations of *Ae. albopictus* from Mexico and Texas relative to the susceptible control strain. The Col. 5 Febrero and WCC strains had the highest resistance levels observed in the field populations from our survey, but the RRs were barely 2-fold greater than the control strain. The results of this study seem consistent with others that have looked

Fig. 5. Allele-specific PCR to detect the F1534C genotypes in the *vgsc* of *Ae. albopictus*. (A) Melting curve peaks of the control DNA templates. A single 80°C peak corresponds to the Phenylalanine (F/F) wild-type homozygote. An 85°C single peak corresponds to the Cysteine (C/C)-resistant homozygote. Two peaks at 80°C and 85°C correspond to the heterozygote (F/C). (B) Agarose gel showing the amplicon size of the three genotypes. We used 100-bp ladders in the first and last wells. Homozygote-resistant (C/C) products are 113 bp long, while the homozygote wild-type (F/F) products are 93 bp. Heterozygotes (F/C) show both bands.

at the resistance status of field *Ae. albopictus* mosquitoes, in which they found that the populations either had low levels of pyrethroid resistance or were susceptible to pyrethroids. For example, complete susceptibility to pyrethroid adulticides (deltamethrin and lambda-cyhalothrin) was observed in *Ae. albopictus* collected from West Bengal, India (Bharati and Saha 2017). Recently, susceptibility to type I and type II pyrethroids was observed in *Ae. albopictus* from Malaysia (Ishak et al. 2015), Italy, and Greece (Vontas et al. 2012). In the United States, susceptibility to deltamethrin, phenothrin, and

Fig. 6. Boxplots comparing cytochrome P450 activity between *Ae. albopictus* strains. The boxes represent the interquartile ranges (i.e., range between the 25th and 75th percentiles) and the whiskers that extend from the boxes represent the min and max observed values (excluding outliers, which are represented by the white dots). Black dots denote the means of each strain and the black lines within the boxes are the medians.

prallethrin was observed in *Ae. albopictus* populations collected from New Jersey, Florida, and Pennsylvania (Marcombe et al. 2014) and to permethrin in populations from North Carolina, Florida, California, and Texas (Richards et al. 2017). In Florida, five *Ae. albopictus* strains had low levels of resistance, with RRs ≤ 1.6 -fold (Estep et al. 2018).

In contrast, pyrethroid resistance in *Ae. albopictus* has been reported in several studies. A survey in *Ae. albopictus* collected from Italy and Greece in 2016 found varied levels of resistance to permethrin and α -cypermethrin, but susceptibility to deltamethrin (Pichler et al. 2018). Suspected resistance to deltamethrin was found in *Ae. albopictus* collected from Central Africa in 2007 (Kamgang et al. 2011) and at a later time point, resistance to deltamethrin and permethrin was detected in *Ae. albopictus* from the same region (Kamgang et al. 2017). Permethrin resistance has also been found in *Ae. albopictus* from Thailand (Ponlawat et al. 2005), Sri Lanka (Karunaratne et al. 2013), and Pakistan (Arslan et al. 2016). In China, two urban-sourced populations of *Ae. albopictus* from Hainan Island had high levels of resistance to permethrin,

Table 2. Mean activity values and standard errors (SE) of each enzyme activity assayed for the three *Ae. albopictus* strains

Enzyme assayed	Mean activity and SE by strain			Significantly different comparisons		
	ATMNJ95 ^a (control)	La Delicada ^b (no selection)	La Macha ^c (selected)	ab	ac	bc
Acetylcholinesterase (% inhibition)	86.21 (± 1.08)	91.01 (± 0.65)	87.53 (± 0.94)	*		
Cytochrome P450s ($\mu\text{g cit/mg ptn}$)	0.17 (± 0.0033)	0.20 (± 0.0032)	0.20 (± 0.0046)	*	*	
Alpha-esterases (nmol naphthol/mg ptn/min)	11.12 (± 0.19)	11.75 (± 0.29)	10.78 (± 0.17)			*
Beta-esterases (nmol naphthol/mg ptn/min)	8.10 (± 0.20)	8.19 (± 0.36)	6.85 (± 0.18)		*	*
Glutathione S-transferases (mml/mg ptn/min)	0.77 (± 0.029)	0.86 (± 0.050)	0.68 (± 0.035)			*
P-nitrophenyl acetate ($\Delta\text{ABS/mg ptn/min}$)	2.16 (± 0.057)	2.58 (± 0.11)	1.76 (± 0.080)	*	*	*

ab, ATMNJ95 vs La Delicada; ac, ATMNJ95 vs La Macha; bc, La Delicada vs La Macha.

Pairwise *t*-test with Bonferroni adjustment that was significantly different (*P*-values < 0.017) is indicated with asterisks (*). In total, 40 females from each strain were tested.

beta-cypermethrin, and deltamethrin (RRs ranged from 8.83- to 436.36-fold) (Chen et al. 2016). In Southern China, two populations were resistant to deltamethrin and the resistance was positively associated with a *kdr* mutation they found at the 1534 site of the *vgsc* (Xu et al. 2016).

In Mexico, *Ae. aegypti* is the major vector of arboviral diseases. Control strategies include breeding site removal, larvicide treatment using Abate, and outdoor ULV spraying of insecticides during outbreaks. From 2000 to 2010, pyrethroids were exclusively used for ULV spraying, resulting in widespread pyrethroid resistance among *Ae. aegypti* populations in Mexico (García et al. 2009, Aponte et al. 2013, Flores et al. 2013). Since *Ae. albopictus* was first reported in Tapachula in 2002 (Casas-Martínez and Torres-Estrada 2003) and our last survey conducted in 2016 found both species overlapping in 80% of the 600 larval-breeding sites throughout Chiapas, we expected that *Ae. albopictus* would be subjected to the same insecticide selection pressure as *Ae. aegypti*; however, this was not the case. Instead, we found high permethrin RRs in *Ae. aegypti* collected at Col. 5 de Febrero (67-fold), San Agustín (80-fold), Puerto Madero (70-fold), Huixtla (58-fold), and Motozintla (91-fold) (unpublished data) but almost complete susceptibility in *Ae. albopictus*. Interestingly, other studies have found similar results. For example, one study found that 21 *Ae. aegypti* strains from Florida were resistant to permethrin with the RRs ranging from 6- to 61-fold, while the five *Ae. albopictus* strains from Florida had very low levels of resistance, with RRs $\leq$ 1.6-fold (Estep et al. 2018). A study in Thailand also found that *Ae. albopictus* were more susceptible to permethrin compared to *Ae. aegypti* collected from the same sites using WHO tube assays with insecticide-impregnated papers (Chuaycharoensuk et al. 2011).

Some studies have found varied levels of resistance when assaying both *Aedes* species from the same regions, but comparatively there appears to be more documented cases of resistance in *Ae. aegypti*, or in some instances where resistance is found in *Ae. albopictus*, *Ae. aegypti* from the same regions have higher levels of resistance, including studies in Thailand (Ponlawat et al. 2005) and Malaysia (Ishak et al. 2015).

The low levels of resistance found in *Ae. albopictus* from Mexico might have resulted from different circumstances, including historical genetic background of the *Ae. albopictus* that invaded Southern Mexico in the early 2000s. Additionally, it is possible that the selection pressure imposed by strategies of vector control failed to target *Ae. albopictus*. Sames et al. (1996) suggested that due to the house configurations within their study sites, mosquitoes in the backyards were protected from the insecticides being sprayed from vehicles on the street, which possibly could help maintain a susceptible population of mosquitoes. Additionally, a recent study conducted in an urban park of São Paulo, Brazil, found that the distributional range of *Ae. aegypti* was more frequently associated with the periphery of the park, which was surrounded by an urban setting; whereas the *Ae. albopictus* were more frequently distributed in the interior portions of the park where there was more vegetation, reaffirming previous reports of habitat preference of the two species and the likelihood that they have dissimilar exposure to insecticides (Heinisch et al. 2019). It would be beneficial to conduct similar studies evaluating the variation of spatial distribution between the two *Aedes* species in Southern Mexico and Texas to see if *Ae. albopictus* are primarily distributed in areas where there is reduced or no exposure to insecticides.

In addition to testing the baseline resistance of field *Ae. albopictus*, we also attempted to select for permethrin resistance by recurrent insecticide exposure of the La Macha strain over several

generations. The RR of the La Macha strain was significantly higher in the F_0 generation compared to the F_3 generation, but there was an unexpected significant decrease of the RR in the F_{11} generation. We were also surprised to find that the RR of the La Macha F_{11} was significantly lower than the RR of La Delicada F_{11} , the nonselected counterpart strain. However, other studies have also reported failed or contradictory results when artificially selecting for resistance in *Ae. albopictus* mosquitoes. One study briefly mentioned that their attempts to select *Ae. albopictus* collected from Florida with permethrin were unsuccessful (Estep et al. 2018). Additionally, Selvi et al. (2010) tried to select for insecticide resistance in *Ae. albopictus* by pressuring fourth instar larvae with malathion. Similar to our study, they observed a decline in resistance with the selected strain (Selvi et al. 2010). For our study, we pressured the La Macha strain a total of five times, starting with the F_3 generation, and selected every other generation up to F_{11} . Perhaps if we were to continue to select the mosquitoes over enough generations and allow the homozygous-resistant individuals (if any are present) to accumulate in the population, the resistance would increase. Future studies would need to confirm this possibility. An additional explanation for the change of permethrin susceptibility could be that the selection pressure inadvertently caused inbreeding within our population. Inbreeding can result in populations with higher proportions of individuals homozygous for deleterious or lethal recessive alleles (Charlesworth and Willis 2009).

Several groups have claimed to successfully select *Ae. albopictus* for low levels of permethrin resistance, based on their comparisons to field-resistant populations. For example, *Ae. albopictus* larvae from Malaysia were selected with the permethrin LC_{50} at every generation, resulting in RRs ranging from 1.90- to 2.20-fold in the F_5 generation (Wan-Norafikah et al. 2013). Also, a laboratory permethrin-treated strain from Kuala Lumpur, Malaysia developed RRs of 3.61- and 3.53-fold. These studies suggest difficulties to artificially select resistant *Ae. albopictus* in the lab.

The low permethrin RRs in the field *Ae. albopictus* from our study are consistent with the lack of *kdr* mutations or elevated enzyme activity usually associated with pyrethroid resistance. Specifically, we screened for the F1534C mutation of the *vgsc* gene in *Ae. albopictus* from the 11 collection sites in Chiapas and from the La Macha, La Delicada, and ATMNJ95 strains. All 700 individual mosquitoes screened in this study carried the wild-type genotype (F1534/F1534). It is possible that the mutations are present in our populations but are in such low frequency that we were unable to detect them. This contrasts with *Ae. aegypti*, in which the F1534C mutation frequency has increased over the last 18 yr and has reached fixation in multiple populations from Southern Mexico as a result of permethrin resistance selection (Vera-Maloof et al. 2015). So far, only three mutations of the *vgsc* have been reported in *Ae. albopictus* from the Americas. The I1011M/V mutations were detected in *Ae. albopictus* from Haiti (McAllister et al. 2012) and the F1534C was reported in *Ae. albopictus* from Brazil (Aguirre-Obando et al. 2017). Interestingly, most of the mutations that have currently been reported in *Ae. albopictus* have occurred in populations from Asia (Kasai et al. 2011, 2019; Chen et al. 2016; Xu et al. 2016; Gao et al. 2018; Li et al. 2018; Zhou et al. 2019), which is where *Ae. albopictus* originated from.

We investigated the enzyme activity between the permethrin-selected strain (La Macha F_{11}), the nonselected counterpart strain (La Delicada F_{11}), and the susceptible control strain (ATMNJ95). Mean activities were significantly higher in the nonselected strain relative to the permethrin-selected strain, except for P450s, in which they had the same levels of activity. The mean activity of P450s was

significantly higher among the permethrin-selected and nonselected Mexico strains compared to ATMNJ95. It is possible that the elevated (and equal) P450 activity is due to the similar genetic background relative to the ATMNJ95 laboratory strain. Although it is possible that P450s do confer resistance in *Ae. albopictus*, further studies using assays with synergists, such as piperonyl butoxide (PBO), which is an inhibitor of P450s (Liu 2015), would help confirm if P450s are actually conferring resistance in *Ae. albopictus*.

This study has provided valuable information regarding the status of permethrin resistance in *Ae. albopictus* from Mexico and Texas; however, there is still much to be learned about insecticide resistance in this species. For instance, it is intriguing that current strategies of vector control targeted against *Ae. aegypti* in Mexico have resulted in the selection of resistance mechanisms in *Ae. aegypti*, but not in the closely related and sympatric species, *Ae. albopictus*. Conducting future screening of pyrethroid susceptibility in *Ae. albopictus* from Mexico and Texas (and other regions around the world) and evaluating possible evolution of resistance-conferring mechanisms are indispensable to develop effective strategies to control these important vectors of arboviruses.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

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