ARE DETOXIFICATION GENE MUTATIONS ASSOCIATED WITH INSECTICIDE RESISTANCE IN *AEDES AEGYPTI?*

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Abstract

The widespread disease vector *Aedes aegypti* presents a severe threat to human populations as a carrier of Zika, dengue, yellow fever, and chikungunya viruses. The primary vector control method involves insecticide use; unfortunately, heavy reliance on insecticides has resulted in the proliferation of insecticide resistance. In addition to mutations in the voltage gated sodium channel that reduce insecticide efficacy, research suggests that metabolic detoxification is a critical resistance mechanism in *A. aegypti*. Using high-throughput sequencing (HTS), previous research (Saavedra-Rodriguez et al., 2019) identified genome-wide polymorphic sites that were significantly associated with deltamethrin resistance in one *A. aegypti* population from southern Mexico named Viva Caucel. In the present study, we used Sanger sequencing to validate the existence of single nucleotide polymorphisms (SNPs) in six detoxification genes identified in the HTS study. These SNPs were located in genes encoding for an oxidation-reduction enzyme (Aldox 10391), an esterase (CCEae2B) and four cytochrome p450 oxidases (CYP4H33, CYP325M2, CYP325G3, CYP325L1). We genotyped knockdown-resistant and susceptible Viva Caucel individuals using allele-specific PCR melting curves to quantif each SNP s association with resistance. The HTS and allele-specific PCR methods reported similar allelic frequencies of resistant versus susceptible phenotypes, confirming the value of HTS for estimating allelic frequencies at polymorphic sites and for detecting mutations putatively associated with insecticide resistance. We subsequently examined the same SNPs in another population from southern Mexico named 5 de Febrero. The SNP located in CYP325L1 was significantly associated with resistance in both populations, suggesting that it likely plays a role in the metabolic resistance mechanism. Evaluating detoxification SNPs is critical to identifying potential genetic markers of insecticide resistance and informing future mosquito control strategies.

Keywords: *Aedes aegypti,* insecticide resistance, deltamethrin, metabolic detoxification, SNP association.

Introduction

Insects are vectors of a multitude of human diseases. In fact, more human deaths occurring between the 17th and early 20th centuries were attributed to pathogens transmitted by insects than all other causes combined (Gubler, 1998). Mosquitoes, specifically, cause higher morbidity and mortality among human populations than any other insects (WHO, 2016). The widespread mosquito species *Aedes aegypti* is the focus of the present study. *A. aegypti* presents a severe threat to human populations as a carrier of Zika, dengue, yellow fever, and chikungunya viruses; dengue alone infects an estimated 390 million people annually (Bhatt et al., 2013; Franklinos et al., 2019; Powell et al., 2018).

Insecticides became a primary tool used in worldwide vector eradication programs in the 1940s; sustained reliance on insecticides since then caused insecticide resistance to become widespread (Benelli et al., 2016; Du et al., 2016; Kawada et al., 2016; Liu., 2015; Raymond et al., 2001). Exposure to insecticides creates a high selective pressure that causes genetic mutations favorably associated with resistance to accumulate over successive generations, resulting in the rapid development of resistance in insect populations (Dusfour et al., 2019, Hemingway & Ranson, 2000; Maciel-de-Freitas, 2014; Ranson et al., 2010).

Pyrethroids are a dominant class of insecticides used for vector control (Du, et al., 2016; Silver et al., 2014; WHO, 2012). Pyrethroids, including permethrin and deltamethrin, are fast-acting and highly lethal among susceptible insect populations, in addition to having low toxicity for humans and other mammals (Bowman et al., 2018; Bradberry et al., 2005; Silver et al., 2014). Pyrethroid insecticides act by binding to the voltage gated sodium channels (vgsc) in neuronal membranes, which slows sodium channel activation and inactivation and prevents action potentials from conducting down the neuron (Bowman et al., 2018; Hemingway et al., 2004). The consequent

blockage of neuronal signaling causes rapid paralysis, or knockdown, and death (Du et al., 2016; Silver et al., 2014).

Using pyrethroids and other insecticides that target the vgsc, including DDT, has resulted in the proliferation of mutations in the *vgsc* gene that cause knockdown resistance (kdr) in many insect species (Liu et al., 2000; Silver et al., 2014; Soderlund & Knipple, 2003; Soderlund & Knipple, 1999). These kdr mutations confer resistance by causing a functional change in the sodium channel that reduces the sensitivity of the target site for pyrethroid binding (Hemingway et al., 2004; Soderlund & Knipple, 2003). Knockdown resistance mechanisms have been studied extensively in mosquitoes in an effort to inform mosquito control strategies and limit the spread of insecticide resistance (Franklinos et al., 2019; Kawada et al., 2015, Zardkoohi et al., 2019).

Documenting the occurrence and rise of kdr mutations is a common method of estimating relative pyrethroid resistance levels among *A. aegypti* populations (Auteri et al., 2018; Bowman et al., 2018; Moyes et al., 2017; Zardkoohi et al., 2019). Five nonsynonymous kdr mutations in the *vgsc* have been confirmed to confer pyrethroid resistance in *A. aegypti* using electrophysiological assays and are hence used as genetic markers of resistance (Du et al., 2016; Saavedra-Rodriguez et al., 2018; Saavedra-Rodriguez et al., 2007; Soderlund & Knipple, 2003). Researchers have found that these kdr mutations differ in geographic distribution, frequency, and relative effect on resistance among different *A. aegypti* populations (Moyes et al., 2017). In addition to the variability of kdr mutations, an emergent challenge with using kdr mutations as markers of resistance is that some of these mutations are nearing fixation in resistant populations (Brito et al., 2018; Ponce Garcia et al., 2009; Sombié et al., 2019; Vera-Maloof et al., 2015). Moreover, kdr mutations are not the only mechanism of insecticide resistance in *A. aegypti* (Seixas et al., 2017; Vontas et al., 2012). As a result, it is of primary importance to characterize the other resistance

mechanisms and identify mutational markers outside the *vgsc* that may be used to assess population resistance levels.

Other mechanisms that are associated with insecticide resistance include metabolic detoxification, cuticle thickening, and behavioral adaptation to limit insecticide exposure (Balabanidou et al., 2018; Kasai et al., 2014; Seixas et al., 2017; Stevenson et al., 2012; Wood et al., 2010; Zalucki & Furlong, 2017). Metabolic detoxification has been identified as a critical resistance mechanism in insect vectors and involves the elevated activity of detoxification enzymes in the insect metabolome that degrade, hydrolyze, or sequester insecticides (Faucon et al., 2015; Hemingway et al., 2004; Marcombe et al., 2009; Moyes et al., 2017). Specifically, the upregulation of several cytochrome p450 oxidase, esterase, and glutathione transferase enzymes has been associated with higher levels of insecticide resistance in *A. aegypti* (Faucon et al., 2015; Mourya, et al., 1993; Strode et al., 2008). However, the role of metabolic detoxification in conferring insecticide resistance is poorly understood in comparison to the kdr mechanism; unlike the kdr mutations in the *vgsc,* mutational markers clearly associated with resistance in *A. aegypti* have not been identified in metabolic detoxification genes (Faucon et al., 2015; Hemingway et al., 2004; Kasai et al., 2014). Detoxification gene markers are required in order to accurately assess the role that metabolic detoxification plays in conferring resistance (Dusfour et al., 2019).

The increased affordability of genome-wide sequencing technologies has provided a promising new method for pinpointing specific mutations in detoxification genes that may be associated with insecticide resistance (Dusfour et al., 2019; Faucon et al., 2015; Saavedra-Rodriguez et al., 2019). High-throughput sequencing (HTS) is used to sequence hundreds of millions of DNA molecules in a single sample; polymorphic sites can be detected throughout the genome by comparing DNA sequences of multiple individuals (Churko et al., 2013). A recent

study by Saavedra-Rodriguez et al. (2019) used HTS technology to identify polymorphic sites associated with deltamethrin resistance in genomic samples from a highly resistant *A. aegypti* population collected in southern Mexico. The researchers compared allelic frequencies between knockdown-resistant and susceptible phenotypic groups in the Viva Caucel population for each polymorphic site and detected hundreds of single nucleotide polymorphisms (SNPs) in detoxification genes that were significantly associated with the resistant phenotype. These candidate detoxification SNPs require further study to investigate their potential roles in conferring resistance, patterns of inheritance, and their viability as genetic markers of deltamethrin resistance.

The first objective of my research was to evaluate the accuracy of the high-throughput sequencing method used by Saavedra-Rodriguez et al. (2019) to identify polymorphic sites associated with deltamethrin resistance. We used Sanger sequencing to verify the existence of selected SNPs located in six detoxification genes and developed allele-specific PCR systems to genotype resistant and susceptible Viva Caucel mosquitoes at the candidate SNP sites. We computed allelic frequencies for each phenotypic group and compared them to the frequencies estimated using the HTS method. The second objective of the present study was to determine whether the six candidate detoxification SNPs we selected were also associated with deltamethrin resistance in another *A. aegypti* population. We used the same allele-specific PCR systems to genotype individuals from a second deltamethrin-resistant population collected in southern Mexico, 5 de Febrero. We compared allelic frequency trends for each detoxification SNP observed in 5 de Febrero and Viva Caucel, aiming to identify detoxification SNPs involved in the metabolic resistance mechanism that are potential genetic markers of deltamethrin resistance. Having verified detoxification markers would provide diagnostic tools to improve the efficacy of strategies used to mitigate the spread of resistance in *A. aegypti* populations.

Materials and Methods

Part 1: Verifying Detoxification SNPs in Viva Caucel and Their Involvement in Resistance

Selecting Detoxification Substitutions for Further Study

We selected SNPs in detoxification genes to test in the present study based on the findings from Saavedra-Rodriguez et al. (2019), in which researchers identified polymorphic sites throughout the *A. aegypti* genome associated with deltamethrin resistance. They used a highly resistant field population collected in southern Mexico called Viva Caucel (see Appendix A). Resistant and susceptible phenotypes in Viva Caucel were separated using a 3 g discriminating dose of deltamethrin, and genomic libraries were formed for each phenotypic group by pooling DNA extracted from 25 individuals. These DNA libraries were subsequently examined using high-throughput sequencing (HTS), which allowed researchers to estimate allelic frequencies at each polymorphic locus. The researchers performed chi square tests for heterogeneity to identify the SNPs that had significantly different allelic frequencies between the resistant and susceptible groups; these SNPs were considered to be putatively associated with deltamethrin resistance. A number of SNPs that surpassed the threshold for significance were located in detoxification genes.

We chose six of these significant detoxification gene SNPs to verify their association with deltamethrin resistance in the Viva Caucel population. The SNPs were located in genes encoding for an oxidation-reduction enzyme (Aldox 10391), an esterase (CCEae2B), and four cytochrome p450 oxidases (CYP4H33, CYP325M2, CYP325G3, and CYP325L1) in chromosomes two and three. We prioritized non-synonymous substitution SNPs that were most highly differentiated between resistant and susceptible groups (Saavedra-Rodriguez et al., 2019). We also selected SNPs located in regions with low genetic variability (having few nearby polymorphic sites), which increased the likelihood of sequencing primers successfully targeting the candidate SNP.

Designing Sequencing Primers and Detecting SNPs

We verified the existence of these six detoxification SNPs by designing primers that targeted each SNP site for Sanger sequencing (Table 1). Primers were designed using Primer Premier and the *A. aegypti* AaegL5 reference genome (Matthews et al, 2018). Each primer pair produced <500 base pair segments containing the target polymorphic site (Table 1). For each SNP, we sequenced three resistant and three susceptible Viva Caucel mosquitoes. As a negative control to confirm the absence of the polymorphism in a susceptible population, we also sequenced three individuals from the lab-raised susceptible New Orleans population at each SNP site. The allele found in the *A. aegypti* AaegL5 reference genome at each SNP site was defined as the reference allele (Matthews et al., 2018); the alternative allele found in the field population (Viva Caucel) was defined as mutant .

DNA samples were amplified in PCR reactions using 12.5 µl of GoTaq Green Master Mix (Promega, Madison, WI), 11.4 µl of deionized water, 0.05 l of forward and reverse primers (at 50pmol/ l), and 1.0 l of DNA. The PCR reactions were run in 0.2 mL tubes in a BioRad MyCycler (BioRad, Hercules, CA) using a program consisting of a denaturation step at 95°C for 5 minutes, followed by 33 cycles of 95°C for 30 seconds, 52°C for 30 seconds, and 72°C for 45 seconds, with a final extension at 72°C for 5 minutes. Samples were stored at 4°C. PCR products were verified using gel electrophoresis with 2% agarose gels run at 85 volts for 30 minutes and purified using the MinElute Purification Kit (Qiagen, Cat. No. 28004/Hilden, Germany). DNA concentrations were measured using a NanoDrop spectrophotometer and prepared for Genewiz Sanger sequencing. The six Viva Caucel sequences and three New Orleans sequences were aligned and assessed for quality using the Geneious software (Biomatters Inc., Newark, NJ).

Table 1. Candidate detoxification gene SNPs validated by Sanger sequencing of Viva Caucel individuals.

Gene	Chromosome	Gene ID	Target Residue	Sequencing Primers	Amplicon Size
Aldox10391	2	LOC5573282	Q642H	CCCAAACCATCGTGTACCTT GCTGTCCTGAATTGTGCTCC	362
CCEae2B	2	LOC23687973	I188V	TTTTCAGGACGGTTCTGGTG TGCAGCCAAATAGAGTCACATT	236
CYP4H33	2	LOC5578646	S421P	ACACTTTGGCATAGTCTTTACG ATTGAATCACCTGGCAGAAC	314
CYP325M2	3	LOC5576780	R225L	AGATCGACATACAATTCCGG ATTTGAGAGTAGTGCTCAGC	266
CYP325G3	3	LOC5576789	F63I	CTTCCAATCATCGCTTACCT GCTCGTCCTATATTCTTCCC	458
CYP325L1	3	LOC5575358	T374A	TACTGGAAGAGTTCAATG CGCATACGGATACTGCTCACG	299

Note. The forward primer is noted first for each SNP, followed by the reverse primer.

Genotyping Viva Caucel DNA Samples at Each SNP Site

In order to assess the accuracy of the HTS method used in Saavedra-Rodriguez et al. (2019) in estimating allelic frequencies, we developed allele-specific PCR melting curve systems to genotype the same 50 resistant and 50 susceptible Viva Caucel individuals used in the HTS method at each detoxification SNP site. Allele-specific PCR requires a common reverse primer and two forward allele-specific primers with different tails to amplify two allele-specific PCR products of different lengths (see Appendix B). One forward primer is designed with a short GC tail at the 5 end, and the second primer targeting the alternative allele contains a long GC tail (Appendix B). PCR products constructed from the high GC-content primer dissociate at a higher temperature than the primer with low-GC content, which allows for identification of alternate genotypes. The absorbance intensity of the florescent dye used to track the progress of PCR product formation increases upon double stranded DNA dissociation, which produces a peak corresponding to the melting temperature of the PCR product (Strayer, 2012). Heterozygous individuals produce two peaks upon subjection to the melting curve.

The allele-specific PCR reaction required 10 µl of iQ Sybr Green Mix (BioRad, Hercules, CA), 9.7 l of deionized H20, 50 M of each allele-specific primer, 100 M of the third primer, and 1 l of DNA. PCR products were subjected to incremental increases in temperature by placing samples in a real-time thermocycler. Melting curve analyses were run in a CFX-96 (BioRad) for 3minutes at 95 C, followed b 39 c cles of 10sec at 95 C, 10sec at 60 C, and 30sec at 72 C, with a final melting curve from 65 C to 95 C with increments of 0.2 C ever 10seconds.

Statistical Analyses

Chi square contingency tests were performed to compare allelic frequencies calculated from the melting curve data and high-throughput sequencing read counts for the Viva Caucel population. Chi square 2x2 contingency tables were used to determine whether there was a nonrandom distribution of genotypes for each kdr mutation and detoxification SNP among the alive and dead phenotypes in Viva Caucel. P-values less than 0.05 were considered significant.

Part 2: Testing SNP Association with Resistance in a Second Population

To confirm each detoxification SNP s association with deltamethrin resistance, we tested allelic frequencies among resistant and susceptible groups in another *A. aegypti* population. We selected another deltamethrin-resistant population collected in southern Mexico called 5 de Febrero (see Appendix A).

Mosquito Rearing Conditions

Eggs from the 5 de Febrero F3 generation were hatched in an incubator set at 27 Celsius. Larvae were separated into clear plastic containers filled with tap water. The containers had approximately equal larval densities and were stored in the 27 C incubator. Larvae were fed daily (mixture of 40 grams of liver powder: 400mL of tap water). Viva Caucel individuals were reared in equivalent conditions for the HTS study (Saavedra-Rodriguez et al., 2019).

Estimating the Deltamethrin LC_{50} for 5 de Febrero

We determined the 5 de Febrero populations level of deltamethrin resistance relative to Viva Caucel by establishing the concentration of deltamethrin that killed half of the mosquitoes following a one hour exposure (the deltamethrin LC_{50}). The Viva Caucel LC_{50} was calculated in Saavedra-Rodriguez (2018). We prepared 1 g/ml, 0.1 g/ml, and 0.01 g/ml stock solutions of deltamethrin by diluting deltamethrin in acetone and subjected adult mosquitoes to a range of doses. We prepared bottles for testing by pipetting the appropriate quantity of stock solution into each Wheaton 250 mL glass bottle with 2mL of acetone. After securing the caps, each bottle was shaken vigorously to coat the bottle and cap evenly with deltamethrin. The cap was removed and the bottle placed on a bottle roller for three minutes prior to storing the bottles overnight to dry.

We conducted LC_{50} testing by aspirating approximately 25 two- or three-day old non-blood-fed adult females into each bottle and recording the number of mosquitoes knocked down every five minutes during a one-hour period, in three replicates. Mosquitoes that were incapable of flight were marked as knocked down. We also aspirated 25 adult females into a control bottle treated with only acetone for one hour to ensure that any residual acetone did not affect knockdown. Mosquitoes were transferred into one-pint cardboard cups covered with clear netting and placed in a 27 C incubator. The number of knocked down individuals was recorded after a 24-hour recovery period. Using a logistic regression in Q calculator, we calculated a 3.5 g deltamethrin LC_{50} for 5 de Febrero. Using this LC_{50}, we estimated that a 1.5 g dose of deltamethrin would be appropriate for separating resistant and susceptible mosquitoes after a four-hour recovery period (as opposed to a 24 hours).

Bottle Bioassay to Establish Resistant and Susceptible Phenotypes

Similar to the LC_{50} testing, we exposed 5 de Febrero adult females to deltamethrin for one

hour to separate resistant and susceptible individuals in a bottle bioassay. We prepared five bottles containing 1.5 g doses of deltamethrin using the same procedure described for the LC_{50} testing. Approximately 50 3-4 day old non-blood-fed adult females were aspirated into each of the five bottles. After one hour, the alive and knocked down mosquitoes were transferred into separate one-pint cardboard cups covered with clear netting and placed into the 27 C incubator for four hours. Individuals that remained active after one hour of exposure to deltamethrin were assigned to the alive phenotype; those that remained knocked down after the four-hour recovery period consisted of the dead phenotype. The mosquitoes in the knocked down cups that recovered during the four hours were separated from the dead group and were not used in the present study. Mosquitoes from the alive and dead phenotypes subjected to the bioassay were frozen in separate 1.5 ml microcentrifuge tubes (see Figure 1 and Table 2).

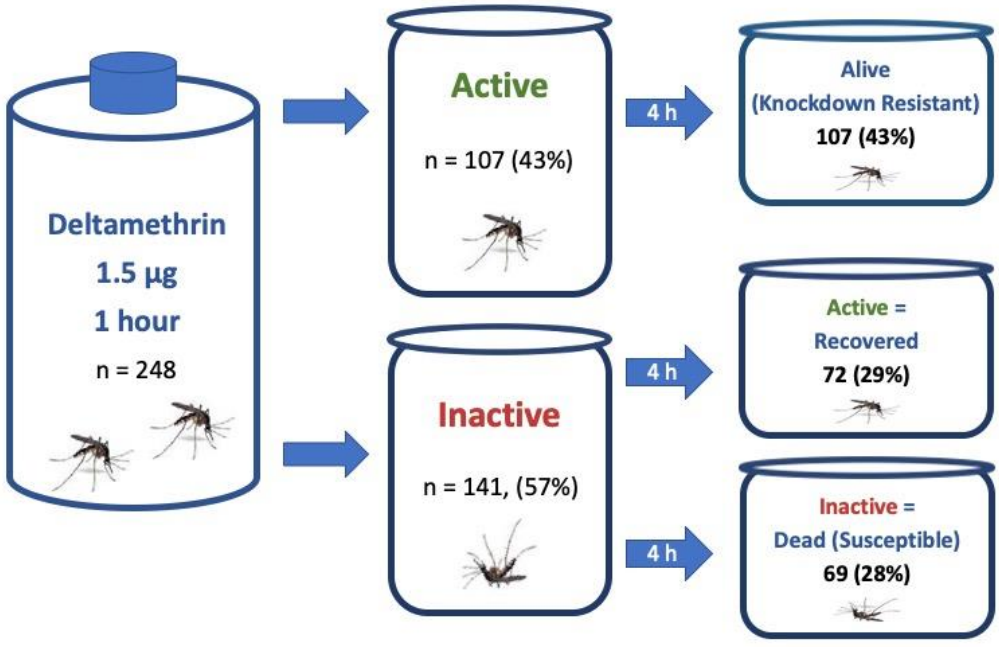

Figure 1. Bottle bioassay procedure used to separate resistant and susceptible mosquitoes; individuals in the recovered group were not used in this study.

Table 2. Results of a 1.5 g deltamethrin bottle bioassay on the 5 de Febrero population.

	Replicate			
	1	2	3	Total
Alive	28	10	10	48
Dead	24	14	23	61
% Survival at 4h	0.61	0.63	0.52	0.59

Note. Replicates four and five are not displayed because individuals in these bottles had a survival rate of over 90% at four hours and were not used in subsequent testing.

5 de Febrero DNA Extraction

DNA was isolated from individuals subjected to the deltamethrin bioassay using the salt-extraction method (Black IV and DuTeau, 1997) and resuspended in 180 l TE (10 mM Tris hydrochloride, 1 mM ethylenediaminetetraacetic acid, pH 8.0). DNA concentrations were measured using a NanoDrop Spectrophotometer and samples were kept and stored at-80 C if concentrations were between 15 and 100 ng/ l.

Genotyping 5 de Febrero DNA Samples to Determine Allelic Frequencies

We genotyped 5 de Febrero individuals at the sites of the six detoxification SNPs using the same allele-specific PCR melting curves as described above for Viva Caucel (Appendix B). A total of 109 5 de Febrero individuals were genotyped at each SNP location from the alive and dead phenotypic groups (48 alive and 61 dead).

As a positive control to confirm differences between resistant and susceptible groups, we also used melting curve primers described in Saavedra-Rodriguez et al. (2018) to genotype 5 de Febrero individuals at the sites of three non-synonymous kdr mutations in the *vgsc* that are strongly associated with insecticide resistance in *A. aegypti.* These three kdr mutations are V1016I, C1534F, and V410L. We compared the 5 de Febrero kdr frequencies to those reported for the Viva Caucel alive and dead phenotypes in Saavedra-Rodriguez et al. (2018).

Statistical Analyses

We tested for nonrandom distribution of genotypes among alive and dead phenotypes in 5 de Febrero for the kdr mutations and detoxification SNPs using chi square 2x2 contingency tables, as described above for Viva Caucel. P-values less than 0.05 were considered significant.

Results

Allele-Specific Melting Curve Systems for Detoxification Gene Substitutions

We designed and optimized allele-specific PCR melting curve systems to genotype mosquitoes at each of the six detoxification SNP sites that we selected for further study. We tested SNPs located in one oxidation-reduction gene (Aldox 10391), one esterase (CCEae2B), and four cytochrome p450 oxidases (CYP4H33, CYP325M2, CYP325G3, and CYP325L1). For each mutation, the amino acid produced by each alternative genotype is denoted by a letter, and the number refers to amino acid residue position in the gene (Figure 2). For example, the mutant TT genotype for the Aldox 10391 mutation produces a tryptophan (H) at amino acid position 642 (Figure 2). For CCEae2B and CYP325M2, the homozygous mutant genotypes (GG and TT, respectively) were not found in the Viva Caucel population; thus, only two peaks are displayed on these figures (Figure 2).

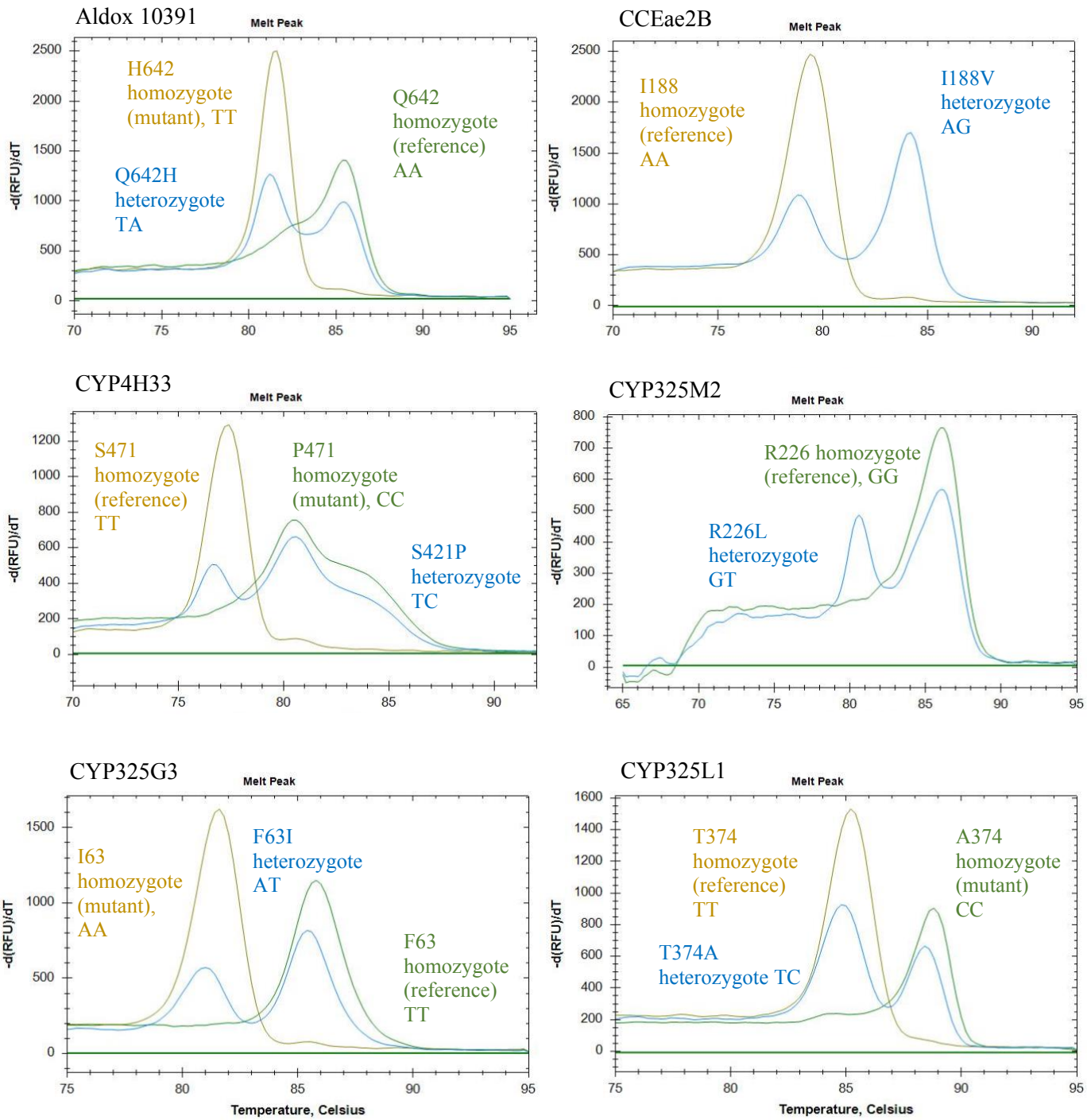

Figure 2. Melting curve peaks display individual genotype patterns for the detoxification SNPs targeted by allele-specific PCR. Figures are titled using the names of the six detoxification genes containing candidate SNPs. Peaks correspond to the reference and mutant genotypes for each SNP and denote the amino acid residues encoded for by each respective genotype.

Validating the High-Throughput Sequencing Method by Melting Curve Analysis

We calculated the mutant allele frequencies at the six detoxification SNP sites using allele-specific PCR in the same 50 resistant and 50 susceptible Viva Caucel individuals used in the HTS study (2019). For the V1016I and V410L kdr mutations located in the voltage gated sodium channel, the frequency of the mutant allele was not significantly different between the melting curve data and the HTS read counts (Table 3). For CYP4H33, we found a significantly higher mutant allele frequency in the dead phenotype using the melting curve technique compared to HTS read counts. The difference between melting curve and HTS results was not significant for the five other detoxification SNPs for both the alive and dead phenotypes. In general, we found that the melting curve and HTS techniques measured similar mutant allele frequencies for both the alive and dead phenotypes (Figure 3).

Table 3. Comparing allelic frequencies of the mutant allele in Viva Caucel using melting curve data and high-throughput sequencing read counts.

Locus/Gene	Phenotype	Melting Curve	Read Counts	2	p-value
kdr V1016I	Alive	0.91	0.89	0.13	0.769
	Dead	0.29	0.16	3.03	0.116
kdr V410L	Alive	0.91	0.82	2.84	0.128
	Dead	0.28	0.21	1.00	0.336
Aldox 10391	Alive	0.37	0.32	0.70	0.475
	Dead	0.28	0.20	1.50	0.250
CCEae2B	Alive	0.02	0.00	1.80	0.485
	Dead	0.10	0.16	1.64	0.264
CYP4H33	Alive	0.33	0.36	0.15	0.770
	Dead	0.15	0.04	5.86	0.034
CYP325M2	Alive	0.02	0.02	0.03	1.000
	Dead	0.18	0.16	0.19	0.712
CYP325G3	Alive	0.02	0.05	1.40	0.405
	Dead	0.22	0.28	0.77	0.468
CYP325L1	Alive	0.05	0.00	4.88	0.061
	Dead	0.38	0.36	0.03	0.866

Note. The kdr mutation F1534C is not included because the HTS data collected in Saavedra-Rodriguez et al. (2019) did not produce read counts for the F1534C polymorphic site.

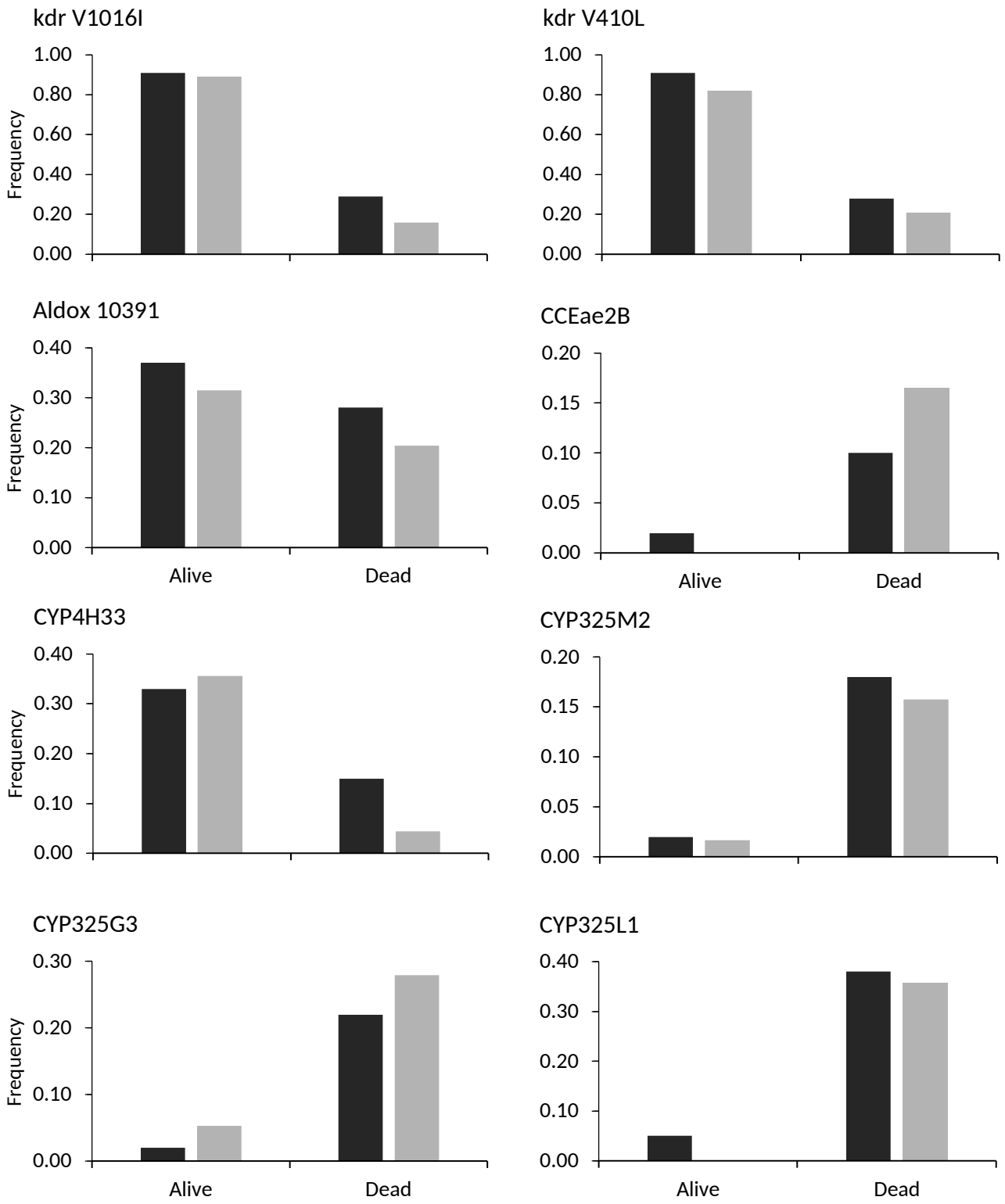

Figure 3. Allelic frequencies of the mutant allele among the alive and dead phenotypes in Viva Caucel calculated by melting curve analysis (dark bars) and high-throughput sequencing read counts (light bars). Results are shown for two kdr mutations and six detoxification gene SNPs. Note that the axes are not scaled uniformly.

Evaluating the Association Between Detoxification Gene Mutations and Resistance

We compared mutant allele frequencies between the alive and dead phenotypes for the six detoxification gene SNPs to allelic frequencies for three kdr mutations that are known to be strongly associated with deltamethrin resistance in order to evaluate the potential association between these detoxification gene mutations and deltamethrin resistance (Saavedra-Rodriguez et al., 2018). We first compared 50 alive and 50 dead Viva Caucel individuals. For the three kdr mutations in Viva Caucel, nearly all individuals in the alive group had the mutant kdr alleles, with significantly lower frequencies in the dead group (Figure 4a). However, we did not observe this relationship for the detoxification genes. Rather, the mutant allele was often more frequent in the dead group than the alive group in Viva Caucel for the detoxification SNPs (Figure 4a).

For the 5 de Febrero population, we compared allelic frequencies for each mutation between 48 individuals in the alive group and 61 in the dead group. The alive group in 5 de Febrero had a higher proportion of the mutant allele than the dead group for the three kdr mutations, which matches the trends found in Viva Caucel (Figure 4b). The frequency of the V1016I and V410L kdr mutations was lower in 5 de Febrero than Viva Caucel, and the C1534F mutation was nearly fixed among both the alive and dead phenotypes in 5 de Febrero (Figure 4). Among the detoxification gene SNPs, the mutant allele frequency was consistently higher in the dead group for the detoxification SNPs, similar to Viva Caucel. The mutant allele was nearly absent in both phenotypes for CCEae2B and CYP325G3 and in the alive group for CYP325M2. Viva Caucel and 5 de Febrero displayed opposite trends for Aldox 10391 and CYP4H33 when mutant allele frequencies between the alive and dead phenotypes. However, both populations had a higher frequency of the mutant allele in the dead group for CYP325L1.

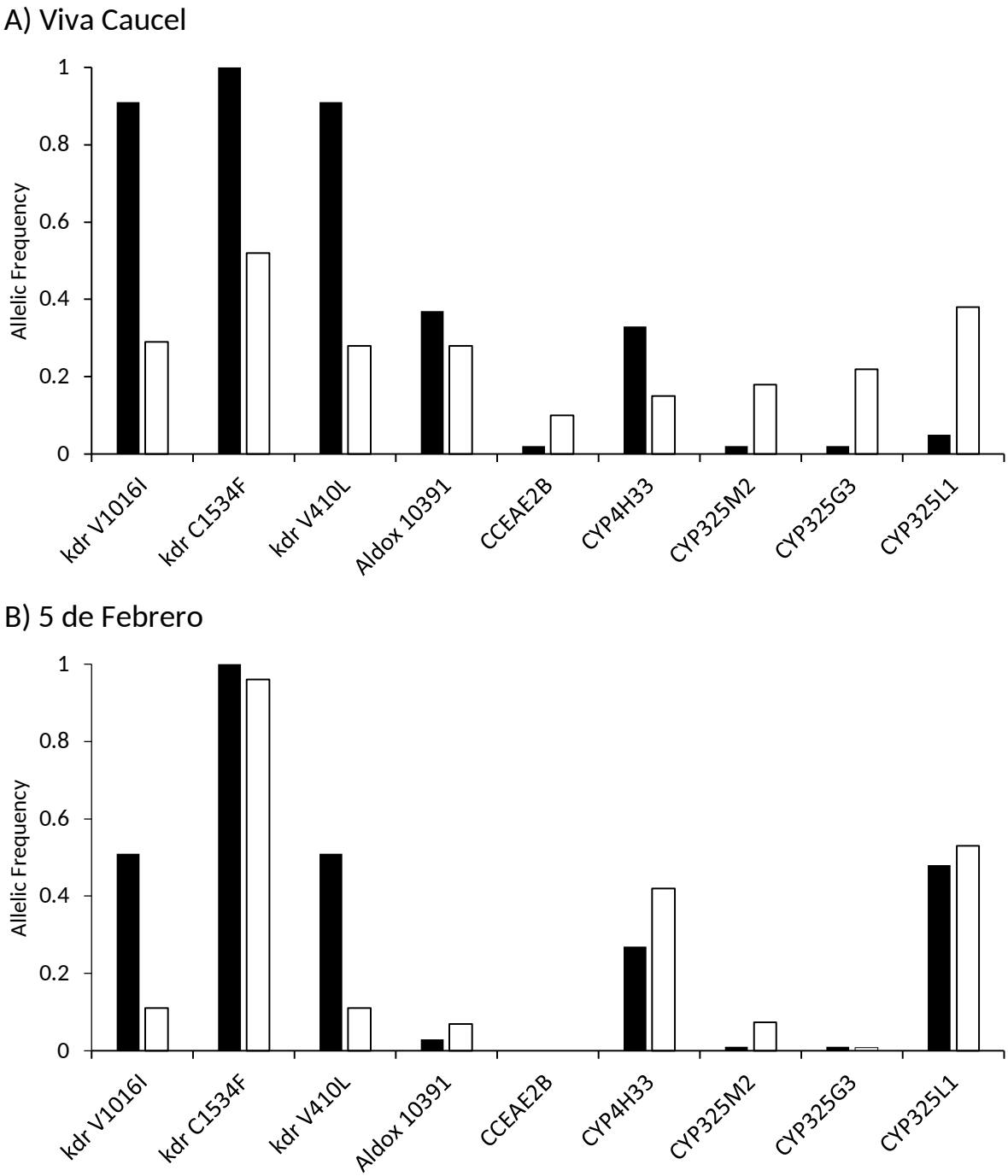

Figure 4. Mutant allele frequencies among the alive (dark bars) and dead (light bars) phenotypes in Viva Caucel and 5 de Febrero calculated by melting curve analysis. Results are shown for three kdr mutations and six candidate detoxification gene SNPs.

We performed chi square analyses on each kdr and detoxification gene mutation to evaluate whether the distribution of genotypes was significantly different among the alive and dead groups

in the Viva Caucel and 5 de Febrero populations (Tables 4 and 5). For all three kdr mutations, the alive and dead phenotypes had significantly different genotypic frequencies in both populations, with the exception of the F1534C mutation for 5 de Febrero (Table 4).

Table 4. Genotypic frequencies for kdr mutations among the alive and dead phenotypes in the Viva Caucel and 5 de Febrero populations. *P*-values were calculated using a chi square 3x2 contingency table assuming Hardy-Weinberg equilibrium for the distribution of genotypes; significant *p*-values are in bold.

		Viva Caucel		**5 de Febrero**	
Locus	Genotype	Alive n=50	Dead n=50	Alive n=48	Dead n=61
V1,016I	I/I	0.84	0.04	0.19	0
	I/V	0.14	0.5	0.65	0.21
	V/V	0.02	0.46	0.17	0.79
	p	**3.77E-19**		**1.49E-10**	
F1,534C	C/C	1	0.16	1.00	0.92
	C/F	0	0.71	0.00	0.08
	F/F	0	0.12	0.00	0
	p	**2.18E-14**		2.15E-01	
V410L	L/L	0.84	0.02	0.19	0
	L/V	0.14	0.52	0.65	0.23
	V/V	0.02	0.46	0.17	0.77
	p	**1.51E-19**		**4.79E-10**	

Note. Genotypes are denoted by the amino acid residues that alternative alleles encode for at each SNP location. The genotype associated with resistance is listed first for each kdr mutation.

Among the detoxification genes, we found significant differences in genotypic frequencies between the alive and dead phenotypes in five of our six SNPs in Viva Caucel and two SNPs in 5 de Febrero (Table 5). The CYP325L1 SNP was the only detoxification gene mutation in which we found a similar genotypic distribution in both populations, with the mutant T/T genotype significantly more associated with the alive phenotype than the dead.

Table 5. Genotypic frequencies for detoxification SNPs among the alive and dead phenotypes in the Viva Caucel and 5 de Febrero populations. Significant p-values are in bold.

Gene	Genotype	**Viva Caucel**		**5 de Febrero**	
		Alive n=50	Dead n=50	Alive n=48	Dead n=61
Aldox 10391	H/H	0.1	0.12	0	0
	Q/H	0.54	0.32	0.06	0.15
	Q/Q	0.36	0.56	0.94	0.85
	p	2.78E-01		2.66E-01	
CCEae2B	V/V	0	0	0	0
	I/V	0.04	0.2	0	0
	I/I	0.96	0.8	1	1
	p	**5.97E-04**		*	
CYP4H33	P/P	0.08	0.04	0.08	0.20
	S/P	0.5	0.22	0.38	0.44
	S/S	0.42	0.74	0.54	0.36
	p	**2.99E-02**		**3.11E-09**	
CYP325M2	L/L	0	0	0	0
	R/L	0.04	0.36	0.02	0
	R/R	0.96	0.64	0.98	1
	p	**9.34E-04**		4.71E-01	
CYP325G3	I/I	0	0.08	0	0
	F/I	0.04	0.28	0.02	0.02
	F/F	0.96	0.64	0.98	0.98
	p	**1.89E-05**		7.29E-01	
CYP325L1	A/A	0	0.06	0.21	0.28
	T/A	0.1	0.64	0.53	0.48
	T/T	0.9	0.3	0.26	0.23
	p	**6.73E-08**		**1.41E-07**	

Note. A chi square test for the CCEae2B genotypic distribution in 5 de Febrero could not be performed due to the absence of the V/V and I/V genotypes in both the alive and dead phenotypes. The mutant homozygous genotype is listed first and the reference genotype is listed third for each gene.

Discussion

In the absence of effective drugs or vaccines for the majority of vector-borne diseases, the global campaign to mitigate the spread of these diseases relies on vector population control, predominantly through widespread insecticide use (Huang et al., 2017; Kasai et al., 2014; Thomas, 2018; Van den Berg et al., 2012). Pyrethroids, including deltamethrin, are a major class of insecticides used for mosquito control (Amelia-Yap et al., 2018; Silver et al., 2014). While the kdr mechanism is well understood, the role of metabolic resistance and detoxification enzymes in conferring pyrethroid resistance is poorly characterized (David et al., 2013; Faucon et al., 2015; Kasai et al, 2014; Liu, 2015). Moreover, as kdr mutations approach fixation in resistant *A. aegypti* populations, documenting the relative frequencies of kdr mutations has become a less effective strategy for quantifying evolving resistance levels (Brito et al., 2018; Martins et al., 2009; Saavedra-Rodriguez et al., 2019). The aim of the present study was to analyze the relationship between six polymorphic sites in detoxification genes and deltamethrin resistance in two *A. aegypti* populations collected from southern Mexico to identify detoxification SNPs outside of the *vgsc* that could potentially be utilized as markers of pyrethroid resistance.

The first objective of our research was to assess the accuracy of the high-throughput sequencing method used by Saavedra-Rodriguez et al. (2019) to estimate allelic frequencies at polymorphic sites. There are several sources of bias in high-throughput sequencing technology, including inaccurate sequencing in highly variable genomic regions and errors caused by pooling DNA samples from multiple individuals (Lam et al., 2014; Reuter et al., 2015; Ross et al., 2013). Since the HTS method used by Saavedra-Rodriguez et al. (2019) involved DNA samples pooled from 25 mosquitos of the same phenotype (resistant or susceptible), the researchers could not confirm that each individual was sequenced at a particular genomic locus. Rather, it is possible

that some individual samples were sequenced multiple times at a particular polymorphic site, while others were not sequenced, which would have altered estimated allelic frequencies (Lam et al., 2014).

Thus, we genotyped resistant and susceptible Viva Caucel individuals using allele-specific PCR melting curves and calculated allelic frequencies for three kdr mutations and six detoxification SNPs to compare to the HTS data (Figure 3). We concluded that the two methods generally reported results that were not significantly different, which validates the HTS method (Table 3). In a similar study conducted by Faucon et al. (2015), researchers corroborated their HTS data by individual genotyping and found that the two methods reported consistent results. In only one instance did we find a significant difference between the two methods: in the dead phenotype for CYP4H33 (Table 3). The HTS methods use of pooled DNA is a likely source of this unexpected difference. While high-throughput sequencing is useful for identifying genome-wide polymorphic sites, a more precise method involving individual genotyping is necessary to evaluate the specific association between the SNP and the resistant phenotype. In addition, the six melting curve systems that were developed to target polymorphic sites in detoxification genes can be used as a tool for genotyping individuals in other *A. aegypti* populations, which presents the opportunity for further research on the prevalence of these SNPs and their potential associations with resistance in different geographic regions.

Our second objective was to determine whether these six detoxification SNPs were similarly associated with resistance in another deltamethrin-resistant *A. aegypti* population. Achieving this objective first required evaluating the trends among the alive and dead phenotypes in the Viva Caucel population. We expected to find that each detoxification gene SNP had different frequencies of the mutant allele between resistant and susceptible individuals in Viva Caucel, as

these SNPs were identified in the HTS study as being significantly associated with resistance (Saavedra-Rodriguez et al., 2019). We also expected to find a pattern for the six detoxification SNPs that matched the trend clearly observed the three known kdr mutations, for which the alive phenotype had a higher frequency of the mutant allele than the reference phenotype (Figure 4A). We did not observe this result for the six detoxification SNPs. Rather, the mutant allele was higher in the dead phenotype for Viva Caucel in four of the six SNPs (Figure 4A).

A potential explanation for this unexpected pattern among the detoxification SNPs may involve how we identified the reference and mutant alleles. We designated the reference allele as the allele found at each SNP site in the AaegL5 reference genome assembly and expected that the lab-raised New Orleans strain would carry the reference allele (Matthews et al., 2018). The AaegL5 assembly used the offspring of a single mating cross of the *A. aegypti* Liverpool strain (LVP-ib12) originally collected from West Africa in the 1930s (Kuno, 2010). Although a recent study found that the Liverpool strain is genetically related to populations from Asia (Gloria-Soria et al., 2019), the reference allele identified in the genome assembly may be inaccurate for our study because of the temporal and geographical differences between the LIV and the New Orleans strains. The New Orleans strain was collected in Georgia, US in the 1990s. It is possible that it was subjected to insecticides in the field prior to being collected to generate the New Orleans strain. Since then, the New Orleans laboratory strain has not been subjected to insecticides and shows full susceptibility to most insecticide classes. We assumed that New Orleans would have the reference allele at each SNP site as opposed to carrying a mutant allele that is putatively associated with resistance. However, it is possible that a mutant allele present in the New Orleans strain resulted from previous selection in the field that caused it to differ from the LIV strain used for the reference genome.

We suggest that the appropriate reference allele must be the allele found in the Viva Caucel population prior to its subjection to insecticides many generations ago. Considering the multitude of selection pressures faced by Viva Caucel in the field, and the genetic drift and bottlenecks in the lab-raised New Orleans strain, the reference allele in New Orleans may not correspond to the reference in Viva Caucel or other field populations that have been exposed to insecticides. The mutant allele at a SNP site may in fact be what was designated as the reference in this study. As a result, the contradictory finding for a number of SNPs suggesting that the dead phenotype in fact has a higher frequency of the allele designated as mutant in both Viva Caucel and 5 de Febrero is not a valid reason to disregard our findings. Rather, these results warrant further research to confirm which allele is the mutant allele in the Viva Caucel and 5 de Febrero populations at each detoxification SNP site and to determine whether these mutations confer any advantage in the presence of insecticides.

Furthermore, comparing the Viva Caucel and 5 de Febrero populations yielded conclusions about which of the six detoxification SNPs are the most promising potential markers of resistance. The SNP in Aldox 10391 warranted further evaluation in the present study because it was significantly associated with the resistant phenotype in the Viva Caucel population (Saavedra-Rodriguez et al., 2019). We found that the Aldox 10391 SNP did not have significantly different allelic frequencies between alive and dead phenotypes in either population. Thus, it is unlikely that it plays an important role in conferring resistance to deltamethrin. This finding is not unanticipated, however, because the three principal enzyme families associated with the metabolic detoxification of xenobiotics, which are esterases, glutathione s-transferases, and mixed function oxidases, do not encompass redox enzymes such as Aldox 10391 (Hemingway & Ranson, 2000; Liu, 2015; Vontas et al., 2012).

We found contradictory trends for the SNP in CYP4H33 the mutant allele frequency was significantly higher in the alive and dead phenotypes for Viva Caucel and 5 de Febrero, respectively. If this SNP played a direct role in resistance, we would not expect to find opposite patterns in two resistant field populations. In a 2014 study by Reid et al., researchers evaluated the potential role of CYP4H33 and three other CYP4 genes in the metabolic detoxification of pyrethroids in *A. aegypti.* They concluded that all of these CYP4 genes likely played some role in conferring pyrethroid resistance, but the CYP4H33 played a lesser role than other CYP4 genes (Reid et al., 2014). These data align with the conclusion from our study that CYP4H33 is unlikely to be a critical gene in the metabolic detoxification mechanism in *A. aegypti.* Rather, the findings in the present study suggest that this particular CYP4H33 polymorphic site is highly variable within and across populations. It is not a promising marker of deltamethrin resistance, but is a robust neutral marker for population genetics studies.

For the polymorphic sites we tested in CCEae2B, CYP325M2, and CYP325G3, the mutant allele was nearly absent in both the alive and dead groups in 5 de Febrero. The data for 5 de Febrero is thus inconclusive regarding the potential role of these polymorphic sites in conferring deltamethrin resistance. In contrast, the mutant alleles for these three SNPs were significantly more present in the dead phenotype in Viva Caucel. Considering the high genetic variability and plasticity that has been identified among *A. aegypti* populations (Craig et al., 1961; Craig & Hickey, 1966; Estep et al., 2017), it is possible that these SNPs may be associated with resistance in some populations and absent in others. For example, kdr mutations have been found to confer varying levels of resistance in different populations (Hirata et al., 2014; Moyes et al., 2017).

Based on prior research, the SNP in CYP325G3 is the most likely among these three polymorphic sites to clearly play a role in conferring resistance. In the case of CYP325G3, two

studies found evidence of upregulation of this gene in two different pyrethroid-resistant *A. aegypti* populations (Reid et al., 2014; Saavedra-Rodriguez et al., 2011). In contrast, Reid et al. (2014) concluded that the slight upregulation of CYP325M2 in a resistant population was not significant (Reid et al., 2014). Moreover, Saavedra-Rodriguez et al. (2011) found that the CCEae2B gene was downregulated in four *A. aegypti* field populations collected in Mexico and upregulated in a Peruvian population, hence failing to establish a clear link between CCEae2B and the resistant phenotype. Considering the inconclusive findings for these three genes and their respective polymorphic sites, a recommendation for future research is to use the allele-specific melting curve systems designed in the present study to test a wide range of populations for the presence of these SNPs and their associations with the resistant phenotype.

Finally, the SNP located in CYP325L1 is the most promising potential marker of resistance among the six detoxification SNPs tested in this study: the dead phenotype had a significantly higher proportion of the mutant allele in both Viva Caucel and 5 de Febrero. This consistent trend indicates that this particular polymorphic site, and more broadly the CYP325L1 gene, is likely implicated in the deltamethrin resistance mechanism. In addition, a prior study examining an *A. aegypti* population in Puerto Rico estimated that detoxification by cytochrome p450 enzymes, including CYP325L1, was the source of about half of the resistance mechanism (Estep et al., 2017). Specifically, CYP325L1 was one of more than 20 cytochrome p450 oxidases that were overexpressed in this resistant population (Estep et al., 2017).

Overall, the SNPs in the cytochrome p450 enzymes that we tested appear to be most directly involved in the pyrethroid-resistance mechanism. Considering these findings and the plethora of research on *A. aegypti* and other insect species linking cytochrome p450 enzymes to insecticide resistance (Kumar et al., 2002; Li et al., 2007; Reid et al., 2014; Scott & Kasai, 2001;

Stevenson et al., 2012; Vontas et al., 2005), gaining a comprehensive understanding of the candidate SNPs in cytochrome p450 genes should be prioritized in future research. However, identifying polymorphic sites in cytochrome p450 oxidases that are clear markers of resistance in *A. aegypti* is challenging. *A. aegypti* has at least 160 catalytically active p450 genes (Strode et al., 2008). Broadly, this superfamily of enzymes catalyzes a diverse array of reactions involving the synthesis or degradation of endogenous compounds and the breakdown of xenobiotics, including insecticides (Mansuy, 1998). As a result, numerous biological processes activate insecticide detoxification enzymes, meaning that genes encoding for cytochrome p450s implicated in resistance are likely subjected to multiple selection pressures besides insecticide use (Chang et al., 2017). It is possible that mutations in detoxification genes conferring insecticide resistance carry negative fitness effects; researchers have discovered evidence of fitness costs associated with resistance in *A. aegypti* and other vector species (Rivero et al., 2010). While p450s evidently play an important role in insecticide resistance, they are numerous and hard to functionally characterize.

Another potential complicating factor is that many cytochrome p450 genes are located in clusters in chromosomes two and three (Saavedra-Rodriguez et al., 2019; Strode et al., 2008). A number of CYP325 genes that Saavedra-Rodriguez et al (2019) found to be significantly associated with resistance, including CYP325L1 and CYP325G3, were clustered in chromosome three. Gene clustering makes it difficult to pinpoint the specific effect of a single polymorphic site or gene, which is supported by evidence of genetic hitchhiking effects associated with resistance in *A. aegypti* (Saavedra-Rodriguez et al., 2019; Yan et al., 1998). Genetic hitchhiking, or a genetic sweep, occurs when allelic frequencies change in polymorphic sites that are not subject to selection because they are closely linked to a nearby allele that is being selected for (Barton, 2000). It is likely that a number of candidate polymorphic sites in detoxification genes highlighted in

Saavedra-Rodriguez et al. (2019) are not directly linked to resistance, but have altered allelic frequencies due to nearby mutations that do confer resistance; it is difficult to identify which SNPs are directly responsible for insecticide detoxification.

Metabolic resistance appears to require the coordination of many genes, and likely implicates various detoxification gene mutations (Ishak et al., 2017; Ishak et al., 2016; Kasai et al., 2014). The allele-specific melting curve systems developed in this study will be useful for genotyping individuals in other populations to confirm whether any of these SNPs, particularly CYP325L1 and CYP325G3, are consistently associated with the resistant phenotype and may be utilized as mutational markers of resistance. Considering the variable distribution of mutations among different *A. aegypti* populations (Craig et al., 1961; Craig & Hickey, 1966), it may be necessary to identify mutational markers of resistance in detoxification genes that are specific for one or a few field populations. For example, CYP325G3 could function as a resistance marker in Viva Caucel, but clearly would not be an effective marker in 5 de Febrero. Another logical next step for research is to map patterns of polymorphic sites in genes encoding for cytochrome p450s and other detoxification enzymes and compare trends between resistant and susceptible phenotypes. Additional avenues include conducting physiological studies to verify the roles of particular detoxification genes in conferring resistance in *A. aegypti* (Chen et al., 2019).

Resistance is a complex mechanism mediated by multiple pathways. Due to the lack of effective and widely available alternatives to insecticide use for vector control (Van den Berg et al., 2012; Kasai et al., 2014), insecticide resistance will continue to rise among *A. aegypti* populations. Understanding how resistance develops remains a critical priority; such research is necessary for informing future vector control strategies and limiting the threat of mosquito-borne human diseases.

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Appendix A

Aedes aegypti collection sites:

The Viva Caucel population used in this study was collected in 2011 by Universidad Autónoma de Yucat n from Yucat n State in southern Mexico (latitude 20.99827, longitude 89.71827).

The 5 de Febrero poplation was collected in 2018 by the Centro Regional de Investigacion en Salud Publica in Tapachula, located in the state of Chiapas in southern Mexico (latitude 14.920612, longitude -92.258611).

Appendix B

Supplementary Table 1. Primers used for the allele-specific PCR melting curve systems to genotype individuals at the sites of six candidate detoxification gene SNPs.

Gene	Target Residue	Primer	Primer Sequence
Aldox10391	Q642H	A fw	GCGGGCAGGGCGGCGGGGGCGGGGCCGCATTGTTGTAGCCGAAACATCGGA
		T fw	GCGGGCGCATTGTTGTAGCCGAAACATCAGT
		rev	ACAGATTTCGGWGGCAGTTT
CCEae2B	I188V	G rev	GCGGGCAGGGCGGCGGGGGCGGGGCCCCTTCATACCCATATTACCATACAC
		A rev	GCGGGCCCTTCATACCCATATTACCATATAT
		fw	CAATCAACTATCGTTTGGGACC
CYP4H33	S421P	G rev	GCGGGCAGGGCGGCGGGGGCGGGGCCATCGTTCCGGTATAAATTCATCGGG
		A rev	GCGGGCATCGTTCCGGTATAAATTCATCAGA
		fw	ATCCCAGCAGGAACGCARAT
CYP325M2	R225L	G fw	GCGGGCAGGGCGGCGGGGGCGGGGCCAAATCTGGGGACCTAACGCCGACCG
		A fw	GCGGGCAAATCTGGGGACCTAACGCCGATCT
		rev	TGAACGGTATAAACGAGTATGGATG
CYP325G3	F63I	T fw	GCGGGCAGGGCGGCGGGGGCGGGGCCGCTGTTTGCCATTTTGAACGCTT
		A fw	GCGGGCGCTGTTTGCCATTTTGAACGTTA
		rev	TAGGACCGAGCCATCCTCGA
CYP325L1	T374A	G rev	GCGGGCAGGGCGGCGGGGGCGGGGCCATTCGTCCAGACGCACCTCTGC
		A rev	GCGGGCATTCGTCCAGACGCACCTCCGT
		fw	TGAAGGAAGTTATGCGRCTGTG