Loss of pyrethroid resistance in newly established laboratory colonies of *Aedes aegypti*

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Abstract

Background

Resistance to pyrethroid insecticides in *Aedes aegypti* has become widespread after almost two decades of the frequent use of these pesticides to reduce arbovirus transmission. Despite this resistance, pyrethroids continue to be used because they are relatively inexpensive and have low human toxicity. Resistance management has been proposed as a way to retain the use of pyrethroids in natural populations. A key component of resistance management is the assumption that negative fitness is associated with resistance alleles such that resistance alleles will decline in frequency when the insecticides are removed. At least three studies in *Ae. aegypti* have demonstrated a decrease in pyrethroid resistance once the insecticide has been removed.

Methods/Principal findings

The present study aims to evaluate variation in the loss of pyrethroid resistance among newly established laboratory populations of *Ae. aegypti* from Mexico. Eight field collections were maintained for up to eight generations, and we recorded changes in the frequencies of the mutations at the V1,016I locus and at the F1,534C locus in the voltage-gated sodium channel gene (*VGSC*). I1,016 and C1,534 confer resistance. We also examined resistance ratios (RR) with type 1 and 2 pyrethroids.

Conclusions/Significance

We demonstrate that, in general, the frequency of the *Ae. aegypti* pyrethroid-resistance alleles I1,016 and C1,534 decline when they are freed from pyrethroid pressure in the laboratory. However, the pattern of decline is strain dependent. In agreement with earlier studies, the RR was positively correlated with the frequencies of the resistance allele I1,016 and

showed significant protection against permethrin, and deltamethrin, whereas F1,534C showed protection against permethrin but not against deltamethrin.

Author summary

The author is interested in the evolution of genes that confer resistance to insecticides, especially when this evolution affects the binding of insecticides to their target sites. The Voltage-Gated Sodium Channel gene provides an excellent opportunity to understand how mutations at the target site(s) affect the evolution of resistance in many different pest insect species, including *Aedes aegypti*, which is the primary vector of the dengue, yellow fever, Zika and chikungunya viruses.

Introduction

After almost two decades of frequent pyrethroid use for *Aedes aegypti* (L.) control, widespread resistance now exists [1, 2]. Despite this, pyrethroid use continues because these insecticides are relatively inexpensive and have low human toxicity. Resistance management has been proposed as a way to preserve the effectiveness of pyrethroids for *Ae. aegypti* control programs [2]. A key component of resistance management is the assumption that negative fitness is associated with resistance alleles such that the resistance alleles will decline in frequency when the insecticides are removed.

Laboratory strains of *Ae. aegypti* have shown a decrease in resistance once a pyrethroid is removed, thereby suggesting a fitness cost is associated with resistance. To date, three laboratory studies have evaluated the loss of pyrethroid resistance in *Ae. aegypti*. In Taiwan, a permethrin-resistant laboratory strain was maintained for 47 generations under permethrin pressure. Following 15 generations without exposure, a significant decrease was observed in the permethrin resistance ratio (RR) and resistance alleles in the Voltage-Gated Sodium Channel gene (VGSC) [3]. In Brazil, after 15 generations, the frequency of I1,016 decreased from 0.75 to 0.20 [4]. A study in Mexico showed a significant increase in the proportion of knocked-down mosquitoes ten generations after removal of pyrethroid exposure [5] but without a decrease in the frequency of I1,016 and C1,534 mutations in the VGSC. All three studies were done in laboratory cages.

The present study aims to evaluate the loss of pyrethroid resistance from eight collections of *Ae. aegypti* (six field collections from or near the city of Merida and two collections from Tapachula and Acapulco from southern Mexico). These collections were maintained without pyrethroid pressure for eight consecutive generations; during this time, we recorded changes in the frequencies of two mutations—VGSC I1,016 and C1,534—and analyzed the resistance ratios (RR) for permethrin (pyrethroid type 1) and deltamethrin (pyrethroid type 2).

Materials and methods

Aedes aegypti field populations

In 2014, larvae were collected from eight public sites in Mexico. These larvae were reared to adults and then identified as *Ae. aegypti*. The adult females were then blood fed (citrated sheep blood–Colorado Serum Co., Denver Colorado) using an artificial membrane feeder, and the eggs were collected for shipment. The GPS coordinates and name abbreviations of the

Table 1. Collection sites by state and city, geographical coordinates, and site abbreviations.

State	City	Site	Latitude	Longitude	Abbreviation
Guerrero					
	Acapulco	Zapata	16.9049222	-99.8410944	**Acp**
Chiapas					
	Tapachula	Col. 5 de Febrero	14.9204944	-92.2593472	**Tap**
Yucatan					
	Merida	Fco. Montejo	21.0307194	-89.6463639	**Mer1**
		Plan Ayala	21.0135833	-89.6222222	**Mer2**
		U.H. Morelos	20.9420139	-89.5981556	**Mer3**
	Conkal	Center	21.0747917	-89.5199056	**Co**
	Dzitya	Center	21.0623278	-89.6746694	**Dz**
	Acanceh	Center	20.8126083	-89.4505611	**Ac**

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collection sites appear in Table 1. Eggs from Yucatan were collected from three urban sites in Merida and from three villages near Merida. Two additional collections were taken from Tapachula and Acapulco, in the states of Chiapas and Guerrero, respectively.

Establishment and maintenance of field populations

F_1 eggs were sent to Colorado State University. Egg papers were placed in a container with 2 L of tap water to promote development and hatching. Larvae were fed 2 mL of 10% (w/v) liver powder solution every other day. We transferred pupae to cups inside plastic cages for adult emergence. Larvae and mosquitoes were maintained in an incubator at 27–28˚C, 70–80% humidity and a photoperiod of 12 h light:12 h dark. Adults were fed with raisins and allowed access to tap water. Females were offered citrated sheep blood in artificial membrane feeders, every four days, to obtain eggs. Females laid their eggs on moistened filter papers. The eggs were allowed to mature for 48 h before being allowed to partially dry at room temperature; they were then stored in sealed plastic bags. Each collection was split at the F_1 larval stage into three groups of 500 to act as biological replicates. In each subsequent generation, ≈250 adult ♀ and 250 adult ♂ were used to maintain each of the three replicates.

Resistance ratio

We evaluated the changes in the resistance levels by determining the LC_{50} for permethrin and deltamethrin at each of the eight sites in the F_3, F_5, and F_8 generations. Resistance ratios were obtained by dividing the LC_{50} calculated for each site by the LC_{50} calculated for the New Orleans *Ae. aegypti* (NO) susceptible reference strain. This colony was originally collected in New Orleans, Louisiana, by the Centers of Disease Control and Prevention and donated by Dr. William Brogdon. Our laboratory has maintained this colony since 2005 free of insecticide exposure. None of the knockdown resistance (kdr) alleles (I1,016 nor C1,534) are present in NO, and pyrethroid susceptibility is routinely confirmed by bottle bioassays. Commonly, the LC_{50} for permethrin ranges between 0.4 and 0.6 ug/bottle, and for deltamethrin, between 0.09 and 0.15 ug/bottle. We examined the relationship between the RR and the frequencies of the I1,016 and C1,534 alleles. Analyses were performed in the F_3, F_6, and F_8 generations and then for all three generations combined. We used PROC CORR in SAS 9.4 to calculate Pearson's correlation coefficient and to test for significance.

Genotyping V1,016I and F1,534C

DNA was extracted at each generation (F_1-F_8) from individual mosquitoes by the salt extraction method [6] and resuspended in 180 μL of TE buffer. To identify allelic variation, we used the allele-specific polymerase chain reaction (asPCR), followed by generation of a melting curve (CFX-96 BioRad), to identify the genotypes [7–9]. In each of the eight generations, we analyzed three replicates of 50 adult mosquitoes (~25♀ and 25♂) for each of the eight collection sites. Sample sizes were kept intentionally large to minimize the founder effect and genetic drift.

Allele frequencies and linkage disequilibrium analysis

We estimated allele frequencies in each of the eight generations from the genotypic frequencies (resistant allele frequency) = (2 x resistant homozygote + heterozygote) / (2 x sample size). Allele frequencies were compared among the replicates using a 2x3 contingency χ^2 test. Win-BUGS 2.0 [10] with 10^6 iterations was used to calculate the 95% high-density intervals (HDI 95%) around the allele and genotype frequencies. We used ggplot2 in R-3.5.1 to graph the data. In addition, we used LINKDIS [11] and χ^2 tests to calculate the pairwise linkage disequilibrium coefficients (R_{ij}) between alleles at loci 1,016 and 1,534 [12] according to the following:

$$R_{ij} = \Delta_{ij}/\sqrt{((pi(1-pi) + Ci)(pj(1-pj) + Cj))}$$

where Ci = Hobs (i)–p_i^2, Hobs (i) is the observed frequency of the i homozygotes, and

$$\Delta_{ij} = (N/(N-1))\left((T_{ij}/N) - 2p_ip_j\right).$$

N is the sample size, and p_i and p_j are the frequencies of the alleles at locus i = 1,016 and locus j = 1,534, respectively. T_{ij} is the number of times that allele i and allele j occur in the same individual. A χ^2 test was performed to determine if significant disequilibrium exists among all alleles at 1,016 and 1,534. The statistic was calculated and summed over all two-allele interactions as follows:

$$\chi^2_{[1\ d.f.]} = N\sum_i\sum_j \left(\Delta_{ij}^2/p_ip_j\right)$$

Results

Resistance declines in absence of pyrethroids

Permethrin and deltamethrin LC_{50} was determined at generations F_3, F_6 and F_8 (S1 Table). These values were compared against the NO susceptible colony to obtain the resistance ratios (RR). Fig 1 and S1 Table show the resistance ratios (RR) obtained for both pyrethroids at each of the generations evaluated. Permethrin resistance in the F_3 varied from 15-fold to 60-fold in six of the eight sites (Fig 1A). The most resistant colony was Mer3 (60-fold) followed by Acp (45-fold) and Mer3 (39-fold). The remaining colonies had RR between 15-fold to 21-fold. At the F_6 generation, RR ranged between 3-fold to 50-fold. The most resistant sites were Mer3 (50 fold) and Ac (42-fold). Acp, Dz, Mer1 and Mer2 had RR from 17-fold to 25-fold. At the F_8, permethrin RR varied from 2-fold to 28-fold. In general, permethrin RR declined after eight generations in five sites (Ac, Acp, Co, Mer1 and Mer3) whereas two sites did not show changes in RR (Dz and Tap).

Deltamethrin RR ranged from 18-fold to 108-fold in generation F_3 (Fig 1B). The most resistant colonies were Co, Acp and Tap with RR higher than 86-fold. Ac and Dz had RR of 32-fold and 18-fold, respectively. Unfortunately, we did not have enough material to process Mer1, Mer2 and Mer3 at this generation. In generations F_6 and F_8, RR ranged from 3-fold to 27-fold.

A) Permethrin
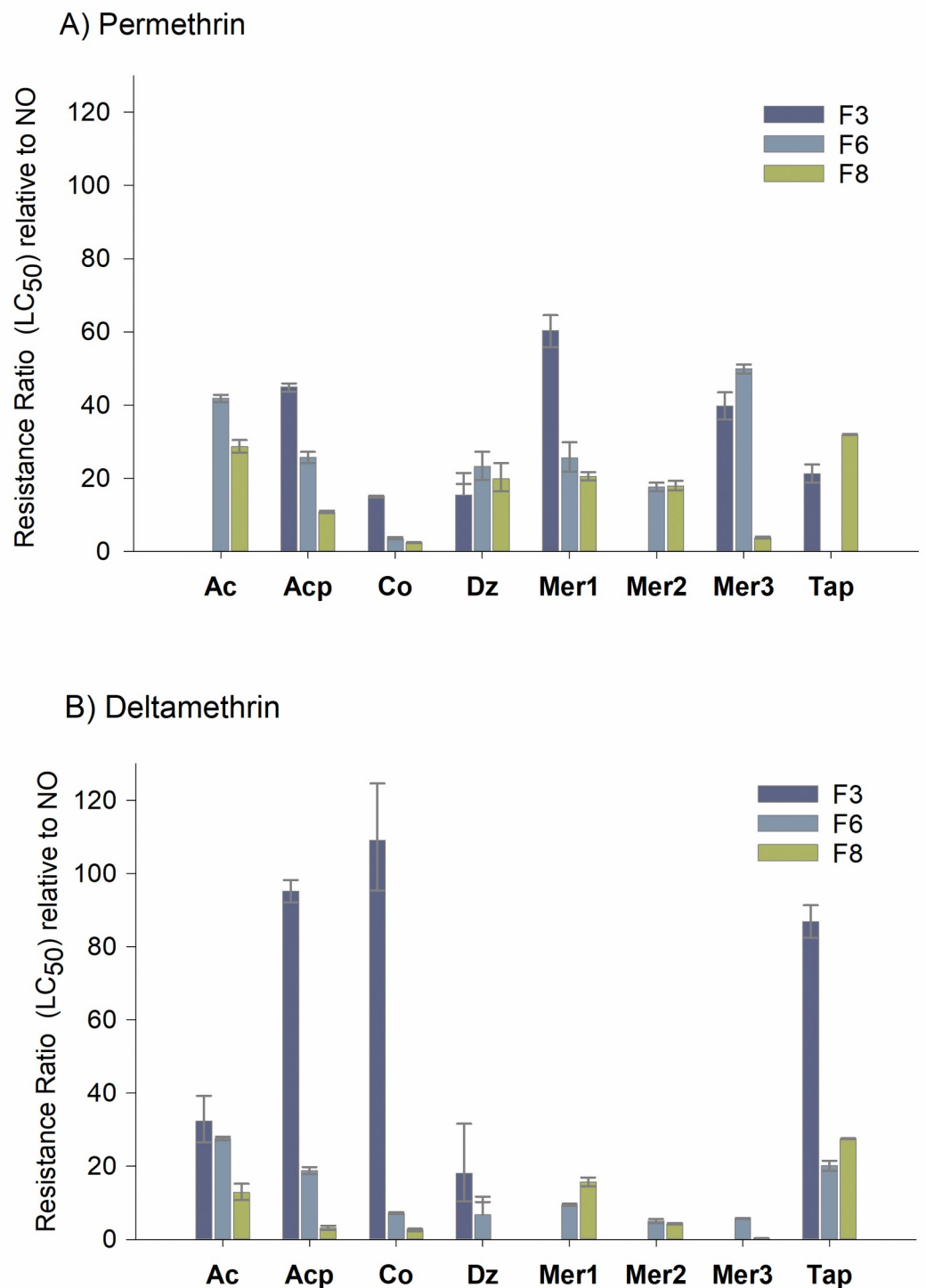
B) Deltamethrin

Fig 1. **A) Resistance to permethrin in generations F_3, F_6 and F_8 in absence of insecticides.** Resistance ratios were calculated by dividing the LC_{50} of our sites by the LC_{50} determined for New Orleans. **B) Resistance to deltamethrin in generations F_3, F_6 and F_8 in absence of insecticides.** Resistance ratios were calculated by dividing the LC_{50} of our sites by the LC_{50} determined for New Orleans.

In general, we observed a decline in RR in five sites (Ac, Acp, Co, Dz and Tap). Mer1 and Mer 2 showed a slight decrease from the F_6 to F_8.

Allele frequency of I1,016 declines in the absence of pyrethroids

We determined the V1,016I and C1,534 genotypes of 9,563 mosquitoes from eight sites in southern Mexico (Table 1) over eight generations in absence of pyrethroids (F_1-F_8). The genotype counts and allele frequencies appear in S2 and S3 Tables, respectively. Allele and genotype frequencies were analyzed separately for each locus. We show a general decline of the resistance alleles in Figs 2 and 4; and the different tendencies of each of the genotypes in Figs 3 and 5.

Fig 2A plots the I1,016 frequencies in all three replicates in the eight different collections over eight generations. Initial allele frequencies in the F_1 varied from 0.35 in Dz to 0.83 in Acp, most of the sites had frequencies ranging between 0.59 to 0.73. In general, the I1,016 allele frequencies were statistically uniform among the three replicates, with five exceptions that are indicated with an asterisk (AcF_2, CoF_2, CoF_8, DzF_2, and $Mer3F_7$) (Fig 2A). Fig 2B plots the mean I1,016 frequencies among all three replicates and the 95% high-density intervals (HDI 95%). The Pearson correlation coefficients between the I1,016 frequencies and the generation number and the associated significance appear at the bottom of each graph. The correlation between the I1,016 allele frequency and generation number was negative in each of the eight collections, though statistical significance varied from highly significant in four out of the eight sites (Ac, Acp, Co, and Mer2) (Fig 2B) to not significant in sites Dz, Mer1, and Tap. The frequency of I1,016 in Co, Mer3, and Tap declined initially and then, surprisingly, increased in the last 3–4 generations. In general, I1,016 declined in frequency over eight generations; however, the rate and pattern of the decline varied greatly among the collections.

Genotype frequencies at 1,016 indicate lower fitness of the resistant-homozygote

To understand the fitness of each genotype, we examined the fate of the three genotypes separately over time (Fig 3). Because the knockdown resistance (kdr) is a recessive trait (only homozygote resistant genotypes survive exposure to pyrethroids), we expect a strong negative fitness cost for homozygote-resistant individuals ($II_{1,016}$) in the absence of pyrethroids. We show in Fig 3C that a decline in $II_{1,016}$ occurs over eight generations, and Table 2 shows negative correlation coefficients for all sites (r = -0.5452, P < 0.0001), confirming a negative fitness for the $II_{1,016}$. Additionally, we show that the susceptible-homozygote or wild-type genotype ($VV_{1,016}$) increased in frequency over the generations with positive correlation coefficients (r = 0.4497, p = 0.0002). This suggests that the susceptible homozygote genotype has greater fitness in the absence of insecticides. Interestingly, we did not observe changes in the frequency of the heterozygotes over time (r = 0.1462, P = 0.2489), indicating that no negative fitness cost occurs for $VI_{1,016}$ in laboratory conditions. Therefore, the resistant allele will prevail in heterozygous individuals for several generations in the absence of insecticides.

Allele frequency of C1,534 declines in the absence of pyrethroids

We obtained the genotypes and allele frequencies for F1,534C in each of the 9,563 mosquitoes for which the V1,016I genotype frequencies had been determined (S2 and S3 Tables). Fig 4A shows the resistant allele C1,534 frequencies in the eight collection sites over eight generations. Initial allele frequencies in the F_1 ranged from 0.67 in Dz to 0.98 in Acp and Tap. The C1,534 frequencies differed between replicates in 15 of the 64 comparisons. The correlation coefficients between the C1,534 allele frequencies and generation number and their significance are
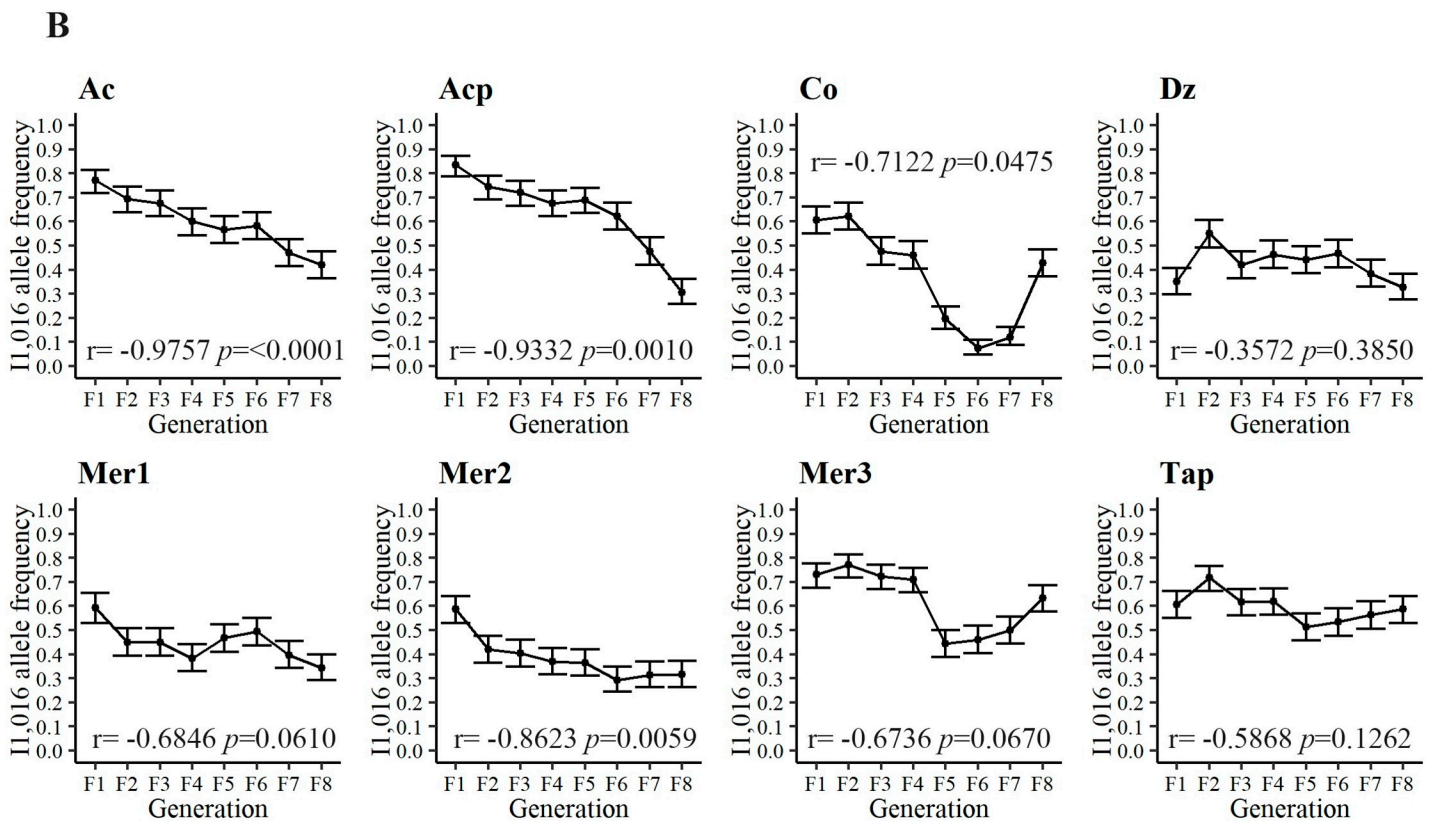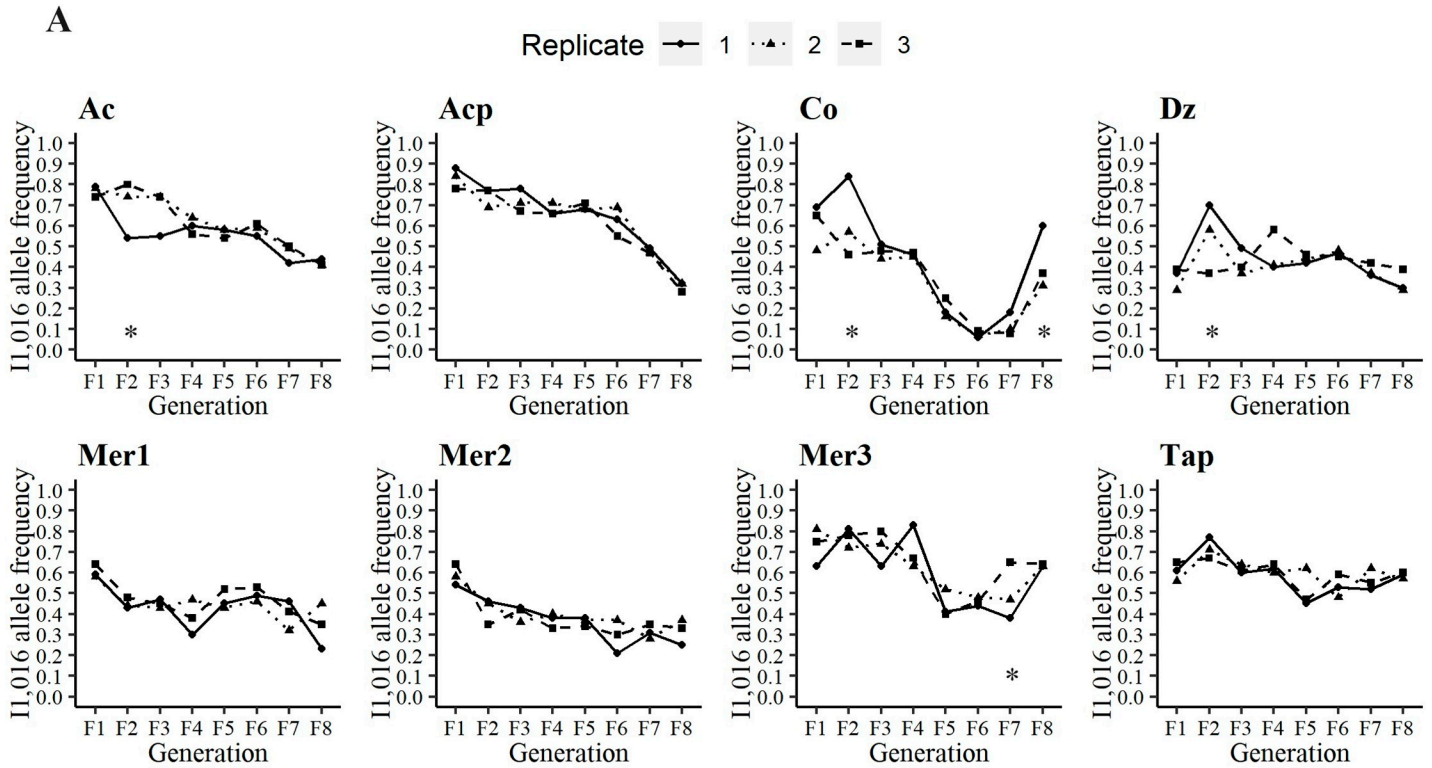
A

Replicate — 1 ···▲··· 2 –■– 3

B

at the base of each of the graphs in Fig 4B. The correlation coefficients between the C1,534 frequency and generation number were negative and significant in four out of the eight collection sites for the F_1 to F_8 generations (Acp, Co, Mer1, and Mer2) (Fig 4B). The correlation was not significant for sites Ac, Dz, Mer3, and Tap. In general, C1,534 declined in frequency over the eight generations; however, the rate and pattern of the decline varied greatly among the collections.

Genotype frequencies at 1,534 indicate lower fitness of the resistant-homozygote

The genotype frequencies at F1,534C are provided in Fig 5, and the correlation coefficients between genotypes and generations are provided in Table 3. Five correlation coefficients for $FF_{1,534}$ were positive, with two being significant; five of the correlation coefficients for $FC_{1,534}$ were positive, with three being significant; and five of the $CC_{1,534}$ were negative, with four being significant. Across all collection sites, a positive correlation ($r = 0.3399$, $p = 0.006$) existed between the homozygote susceptible $FF_{1,534}$ and the generation number. The frequencies of $FC_{1,534}$ did not change significantly over the generations ($r = 0.2078$, $P = 0.0994$), and as expected for a genotype that confers a lower fitness, the $CC_{1,534}$ genotypic frequencies decreased significantly over the generations ($r = -0.3024$, $P = 0.0152$), suggesting a fitness cost associated with the resistant-homozygote genotype.

Linkage disequilibrium

We performed pairwise linkage disequilibrium analyses between the alleles in V1,016I and F1,534C. Table 4 lists the linkage disequilibrium coefficients R_{ij}, χ^2, and the probability value obtained between pairwise loci. R_{ij} is distributed from -1.00 (mutations occur on opposite chromosomes—*trans*) to 0.00 (mutations occur independently) to 1.00 (both mutations on the same chromosome—*cis*); therefore, R_{ij} provides a standardized measure of the disequilibrium.

Alleles segregated in 57 of the 64 collections (Table 4). Forty-seven of the 57 collections exhibited significant linkage disequilibrium, with the R_{ij} values ranging between 0.15 and 0.85 among the collections. This result suggests that the resistance alleles I1,016 and C1,534 occur together more often than expected by independent, random segregation. In general, and in agreement with earlier studies [12, 13], alleles in V1,016I and F1,534C were in linkage disequilibrium.

The four possible haplotypes resulting from V1,016I and F1,534C are shown in Fig 6. Table 5 displays the correlation and the significance between the haplotype frequencies and generations. The frequency of the susceptible $V_{1,016}/F_{1,534}$ haplotype increased over the generations ($r = 0.323$, $p = 0.009$). The frequency of the $V_{1,016}/C_{1,534}$ haplotype remained relatively constant ($r = 0.140$, $p = 0.271$). The frequency of the resistant $I_{1,016}/C_{1,534}$ haplotype decreased over time ($r = -0.516$, $p < 0.0001$) across all collection sites. The frequencies of the $I_{1,016}/F_{1,534}$ haplotypes were consistently low ($r = 0.091$, $p = 0.474$) across generations. This same trend has been noted in two previous studies [5] [13], suggesting that low fitness may occur in any mosquito in which I1,016 co-occurs with F1,534.
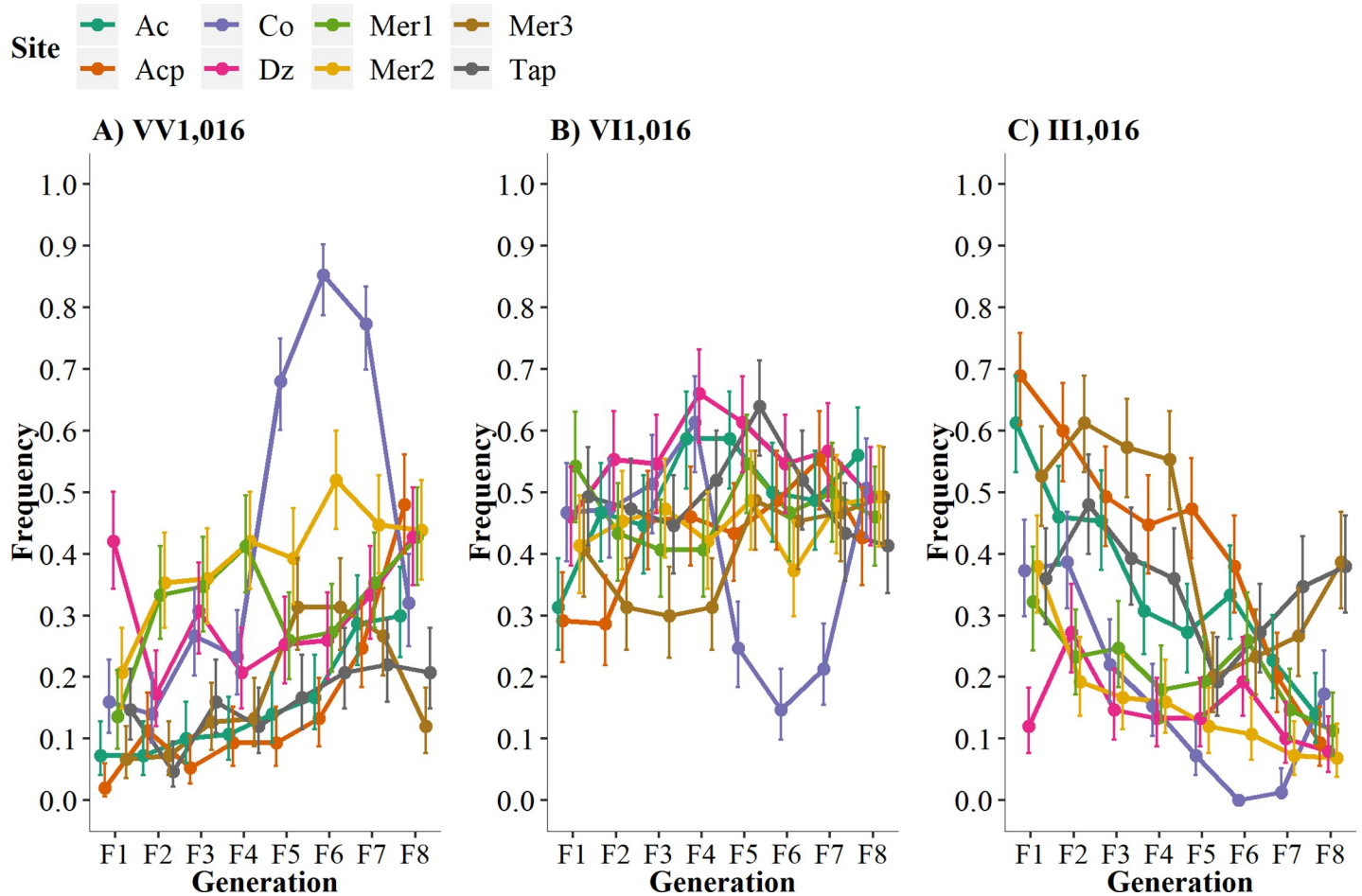
Fig 3. A) Frequency of the resistant allele C1,534 in all three replicates in the eight different collections over eight generations. An asterisk indicates statistical differences among the three replicates. **B) Mean frequency of the resistant allele C1,534 among all three replicates and the 95% high-density intervals (HDI 95%)**. The Pearson correlation coefficient between the C1,534 frequencies and generation number and the associated significance appear at the bottom of each graph.

Temporal analysis of di-locus genotypes

[Fig 7] shows the frequency of the nine di-locus genotype combinations (three genotypes at two loci), and [Table 6] lists the correlation between frequencies of each di-locus genotype and the generation number without pyrethroid exposure. We estimated the frequencies of the nine genotype combinations in 9,563 mosquitoes. However, the di-locus coefficient among the nine graphs was only significant for the wild type susceptible $VV_{1,016}/FF_{1,534}$ and the dual resistant $II_{1,016}/CC_{1,534}$. The correlation between generation number and $VV_{1,016}/FF_{1,534}$ was positive ($r = 0.3376$, $P = 0.0064$), indicating an increase in susceptible di-locus genotypes in the absence of insecticides. The correlation of $II_{1,016}/CC_{1,534}$ was negative ($r = -0.5465$, $P < 0.0001$), indicating a decline. The low frequencies of $VI_{1,016}/FF_{1,534}$, $II_{1,016}/FF_{1,534}$, and $II_{1,016}/FC_{1,534}$ in [Fig 7B, 7C and 7F] are consistent with the hypothesis that low fitness may occur in any mosquito in which I1,016 co-occurs with F1,534. However, [Fig 7E] identifies an exception to this trend. A substantial proportion of the double heterozygote VI_{1016}/FC_{1534} survives to generation F_8.

A

B
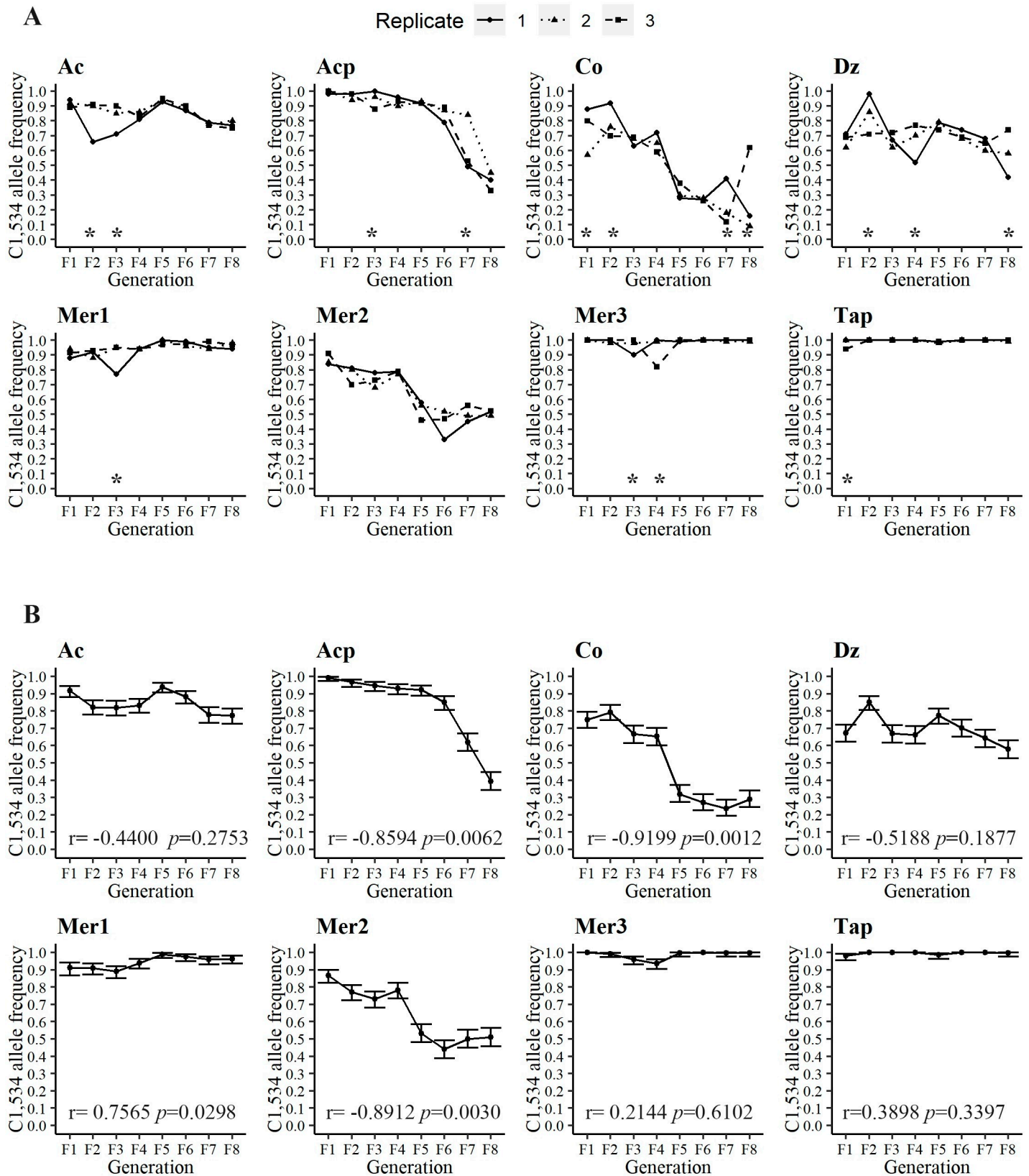
Fig 4. Frequency of genotypes at locus V1,016I over eight generations in the absence of pyrethroids. A) Susceptible-homozygote = $VV_{1,016}$, B) Heterozygote = $VI_{1,016}$, and C) Resistant-homozygote = $II_{1,016}$.

Fig 5. Frequencies of genotypes at locus F1,534C over eight generations in the absence of insecticides. Susceptible-homozygote = $FF_{1,534}$, heterozygote = $FC_{1,534}$, and resistant-homozygote = $CC_{1,534}$.

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Association between resistance alleles and resistance ratios

Pearson correlation coefficients and their significance were calculated between the frequencies of $I_{1,016}$ or $C_{1,534}$ alleles and resistance ratios for permethrin or deltamethrin as determined

Table 2. Pearson's correlation coefficient between the genotypes at locus V1,016I and generations in the absence of insecticides. Susceptible-homozygote = $VV_{1,016}$, heterozygote = $VI_{1,016}$, and resistant-homozygote = $II_{1,016}$.

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

https://doi.org/10.1371/journal.pntd.0007753.t002

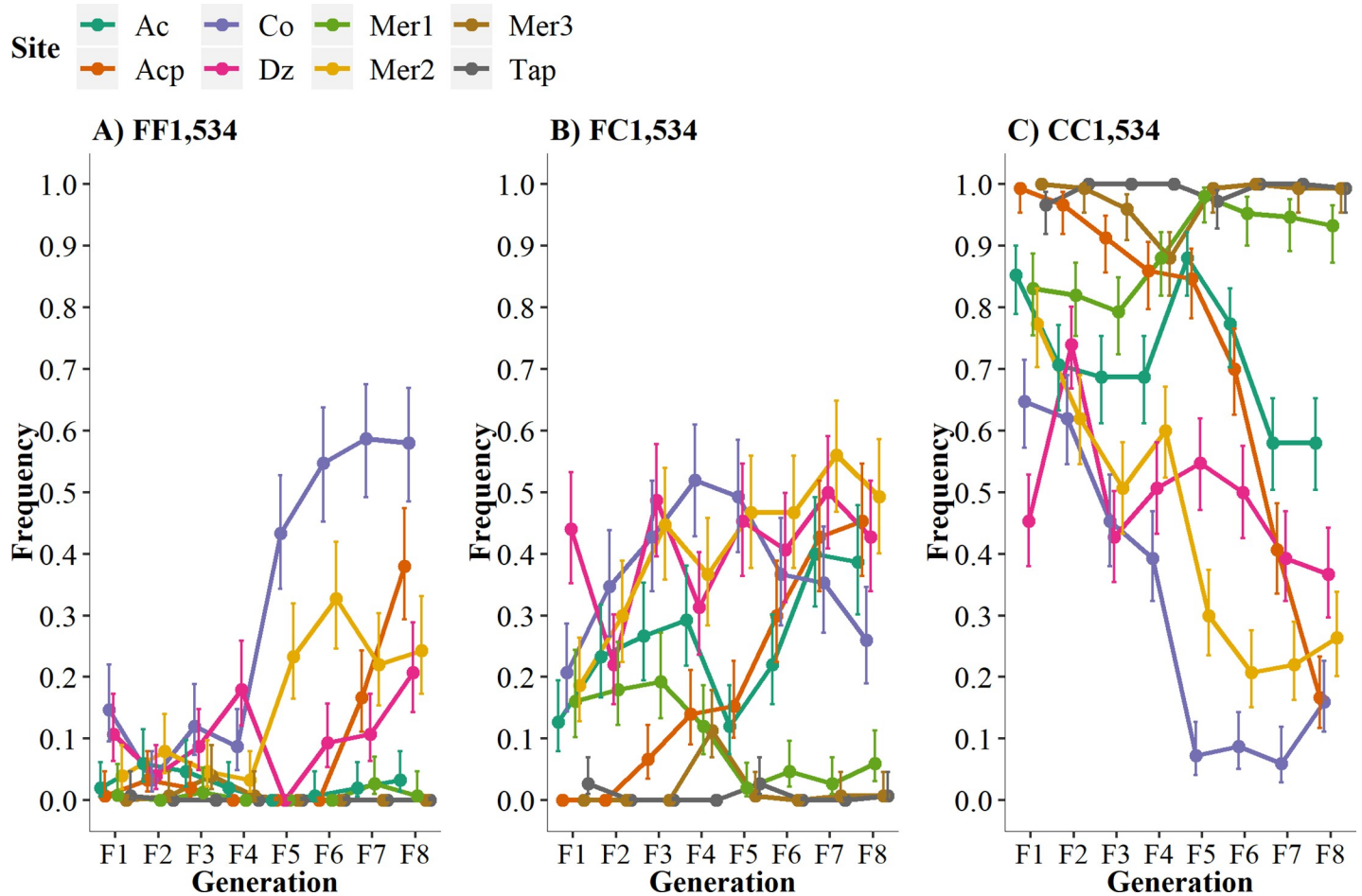**Table 3. Pearson's correlation coefficient between the genotypes at locus F1,534C and generations in the absence of insecticides.** Susceptible-homozygote = $FF_{1,534}$, heterozygote = $FC_{1,534}$, and resistant-homozygote = $CC_{1,534}$.

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

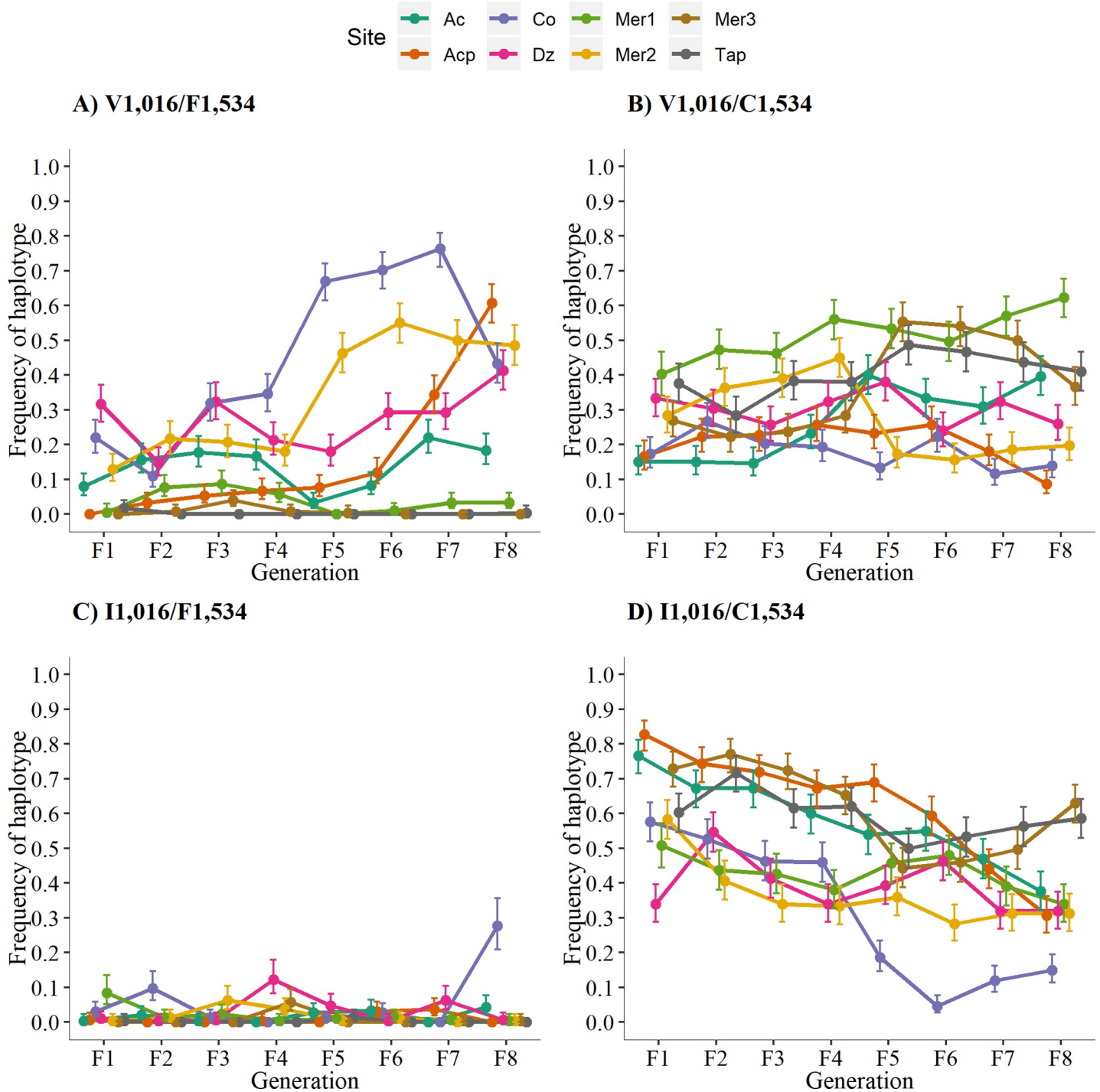https://doi.org/10.1371/journal.pntd.0007753.t003

by bioassay for all eight collections. This analysis was done separately for generations F_3, F_6, and F_8. Table 7 indicates that all correlations were positive in F_3 and F_8, but none were

Table 4. Linkage disequilibrium coefficients between loci 1,016 and 1,534 over eight generations in absence of pyrethroids.

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

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Fig 6. Frequency of the four potential di-locus haplotypes between loci V1,016I and F1,534C over eight generations in the absence of insecticides. Haplotypes: both susceptible alleles in *cis* = $V_{1,016}/F_{1,534}$, susceptible $V_{1,016}$ and resistant $C_{1,534}$ alleles in *cis* = $V_{1,016}/C_{1,534}$, resistant $I_{1,016}$ and susceptible $F_{1,534}$ alleles in *cis* = $I_{1,016}/F_{1,534}$, and resistant $I_{1,016}$ and resistant $C_{1,534}$ alleles in *cis* = $I_{1,016}/C_{1,534}$.

significant. All correlations were positive in F_6, and three were significant. When the results from all three generations were combined, all correlations were positive, and three were significant. Thus, in general, a weak but consistently positive correlation existed between

Table 5. Pearson's correlation coefficient and _p_ value for four possible haplotypes at loci V1,016I and F1,534C. Haplotypes: both susceptible alleles in _cis_ = $V_{1,016}$/$F_{1,534}$, susceptible $V_{1,016}$ and resistant $C_{1,534}$ alleles in _cis_ = $V_{1,016}$/$C_{1,534}$, resistant $I_{1,016}$ and susceptible $F_{1,534}$ alleles in _cis_ = $I_{1,016}$/$F_{1,534}$, and resistant $I_{1,016}$ and resistant $C_{1,534}$ alleles in _cis_ = $I_{1,016}$/$C_{1,534}$.

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

the frequency of the I1,016 or C1,534 alleles and resistance ratios as determined by bioassay (Fig 8).

This correlation also was noted in an earlier study in which I1,016 showed significant protection against permethrin and deltamethrin, whereas F1,534C showed protection against permethrin but not against deltamethrin [14, 15]. The expression of C1,534 in _Xenopus_ oocytes and exposure to both pyrethroids demonstrated that the resistant amino acid substitution in C1,534 is sensitive to permethrin but not to deltamethrin, which is consistent with our results.

Discussion

In this study, we established eight colonies of _Ae. aegypti_ from the field and maintained them in a pyrethroid-free environment in the laboratory for eight generations. We demonstrated that, in general, the frequency of the _Ae. aegypti_ pyrethroid-resistance alleles I1,016 and C1,534 decline when released from pyrethroid pressure in the laboratory (Figs 2 and 4, Tables 3 and 5). However, the pattern of decline appeared to be strain dependent, with some strains showing a steady rate of decline (Ac, Acp, and Mer2 in Fig 2; Acp and Mer2 in Fig 4), some showing a shallow decline (Mer1 and Tap in Fig 2; Acp, Mer2, and Co in Fig 4), and others displaying no net change (Dz in Fig 2; Ac, Mer3, and Tap in Fig 4).

A more surprising result was that, in Co and Mer3, the frequencies of I1,016 increased following a precipitous drop. Likewise, the frequencies of C1,534 in Mer1, Mer2, and Co increased after a drop. Such a change might occur if deleterious or lethal recessive mutations are linked to the susceptible allele that became homozygous through continuous inbreeding. However, this theory fails to explain why I1,016 increased simultaneously in all three replicates of Co.

We do not suggest, nor do we know whether the selection pressure in indoor cage studies is the same as or even correlated with outdoor selection pressure. This study only indicates that the loss of pyrethroid resistance is unlikely to follow a smooth linear or exponential decline for any one of a number of reasons. Nor should we expect the decline in resistance to be consistent among collections. Epistatic interactions between alleles may cause nonlinear trends in allele frequencies. Much depends on the genetic background of each population; some populations could take much longer to lose resistance, while others may do so much more rapidly.

A variety of possibilities exist to explain this variance in the gene frequency trajectories among the collections, but this heterogeneity is unlikely to have arisen from small sample sizes. We analyzed three replicates of 50 adult mosquitoes for each of the eight collection sites.
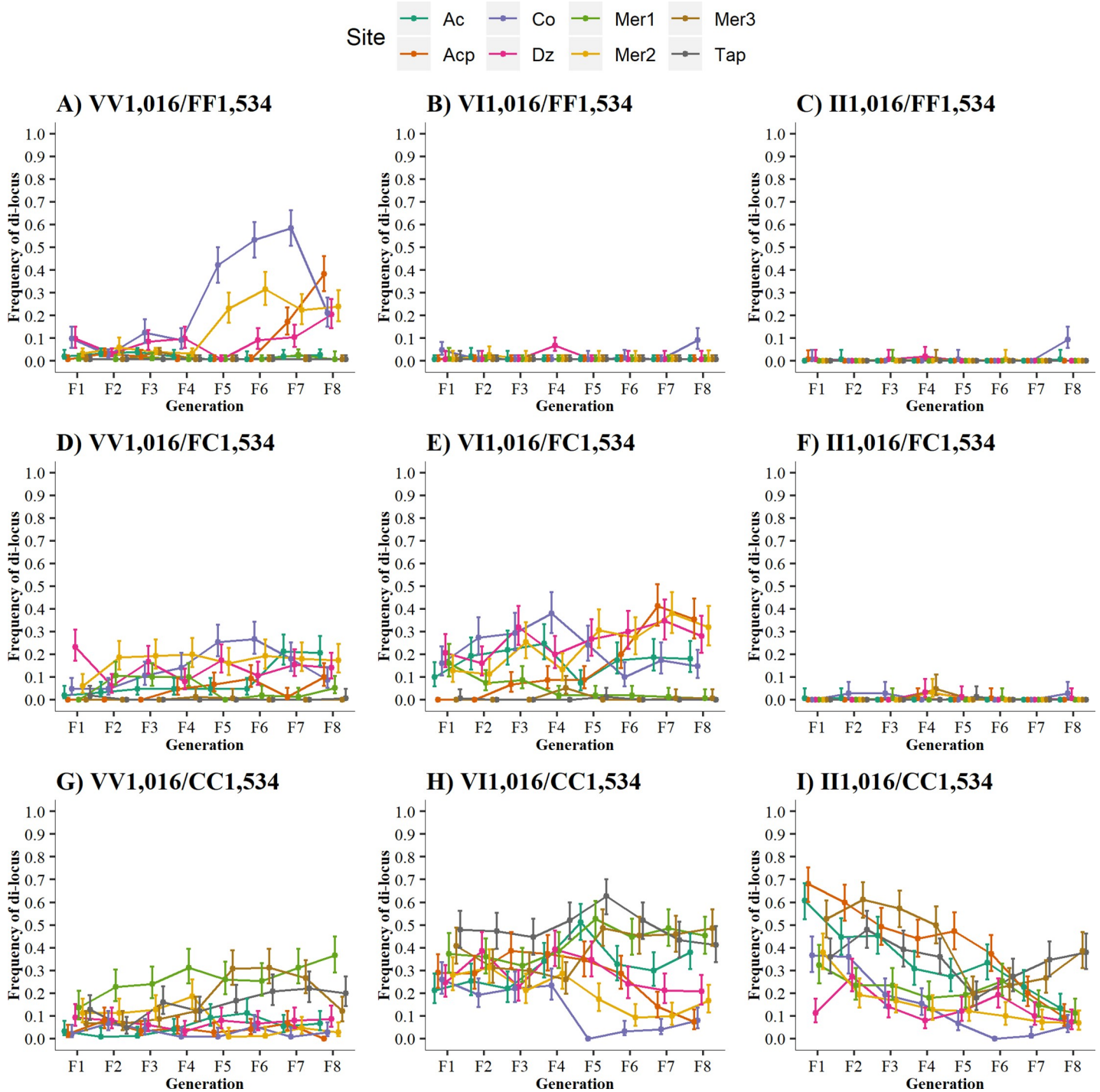
Fig 7. Frequency of the nine di-locus haplotypes over eight generations in the absence of insecticides.

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Furthermore, 500 adults were used to generate the next generation of eggs for each replicate. The 95% HDI remained narrow in all graphs in Fig 2B and Fig 4B.

Initial conditions may affect the shape of the curve. For example, a curve that begins with initial frequencies close to 1 (Mer3 and Tap in Fig 4A) would begin to decline much later than

Table 6. Pearson's correlation coefficient and _p_ value for nine dilocus genotypes at loci V1,016I and F1,534.

	$VV_{1,016}/FF_{1,534}$		$VI_{1,016}/FF_{1,534}$		$II_{1,016}/FF_{1,534}$	
Site	Pearson r	_P_ value	Pearson r	_P_ value	Pearson r	_P_ value
Ac	-0.3225	0.4360	-0.4124	0.31	0.1260	0.7663
Acp	0.7101	0.0484	-	-	-0.5774	0.1340
Co	0.6830	0.0619	0.3135	0.4496	0.5493	0.1585
Dz	0.5151	0.1915	-0.0825	0.8461	-0.3693	0.3679
Mer1	0.2980	0.4734	-0.5774	0.134	-0.2474	0.5546
Mer2	0.8243	0.0118	-0.4124	0.31	0.2474	0.5546
Mer3	-0.2531	0.5454	-	-	-0.0825	0.8461
Tap	-	-	-	-	-	-
Across all	0.3376	0.0064	0.0249	0.8451	0.1370	0.2803
	$VV_{1,016}/FC_{1,534}$		$VI_{1,016}/FC_{1,534}$		$II_{1,016}/FC_{1,534}$	
Site	Pearson r	_P_ value	Pearson r	_P_ value	Pearson r	_P_ value
Ac	0.8132	0.0141	0.1079	0.7993	-0.7559	0.0300
Acp	0.7280	0.0406	0.9162	0.0014	0.1260	0.7663
Co	0.5219	0.1846	-0.4137	0.3082	-0.0563	0.8946
Dz	-0.1429	0.7357	0.6513	0.0802	0.0724	0.8648
Mer1	-0.2719	0.5148	-0.8585	0.0064	0.0000	>0.9999
Mer2	0.4626	0.2485	0.8381	0.0094	-0.0825	0.8461
Mer3	-0.0357	0.9331	0.0494	0.9076	-0.0825	0.8461
Tap	-0.3711	0.3654	-0.2143	0.6103	0.0825	0.8461
Across all	0.2048	0.1046	0.1817	0.1507	-0.0471	0.712
	$VV_{1,016}/CC_{1,534}$		$VI_{1,016}/CC_{1,534}$		$II_{1,016}/CC_{1,534}$	
Site	Pearson r	_P_ value	Pearson r	_P_ value	Pearson r	_P_ value
Ac	0.6439	0.0849	0.5632	0.1461	-0.9390	0.0005
Acp	-0.2323	0.5799	-0.6772	0.0651	-0.9628	0.0001
Co	-0.2698	0.5182	-0.7866	0.0206	-0.9141	0.0015
Dz	0.0398	0.9255	-0.4257	0.293	-0.4131	0.3091
Mer1	0.8522	0.0072	0.7112	0.0479	-0.8105	0.0147
Mer2	-0.6506	0.0806	-0.8032	0.0164	-0.8622	0.0059
Mer3	0.6035	0.1132	0.6312	0.0933	-0.7211	0.0435
Tap	0.7993	0.0173	-0.1480	0.7266	-0.3517	0.3930
Across all	0.1757	0.165	-0.0899	0.48	-0.5465	<0.0001

a curve that begins at 0.6 (Mer1 and Mer2 in Fig 2A). Metabolic resistance may account for much of this heterogeneity. A quantitative trait loci mapping study (QTL) [9] reported that 58.6% of the variation in knockdown could be accounted for by I1,016, but that a number of different QTL located throughout the genome accounted for the remainder of the variation. Saavedra-Rodriguez et al. used the "Aedes Detox" microarray [16] and showed an inverse relationship between I1,016 frequencies and the number of differentially transcribed metabolic genes [17].

Table 5 displays the correlation and the significance between haplotype frequencies and generation. The low frequencies of $VI_{1,016}/FF_{1,534}$, $II_{1,016}/FF_{1,534}$, and $II_{1,016}/FC_{1,534}$ noted in this (Fig 7B, 7C and 7F) and in two previous studies [5] [12] suggest that low fitness may occur in any mosquito in which I1,016 co-occurs with F1,534. This finding, and because C1,534 historically appears before I1,016, suggests that the evolution of the mutations was sequential. If

Table 7. Pearson correlation coefficients and their significance between the frequencies of I1,016 or C1,534 alleles and Resistance Ratios (RR) for permethrin or deltamethrin as determined by bioassays for all eight collections.

| Generation	Permethrin RR	P value	Deltamethrin RR	P value
Locus	I1,016		I1,016	
F3 (n = 9)	0.606	0.1489	0.625	0.184
F6 (n = 9)	0.715	0.0303	0.709	0.032
F8 (n = 9)	0.338	0.3732	0.607	0.083
All (n = 27)	0.588	0.002	0.469	0.021
Locus	C1,534		C1,534	
F3 (n = 9)	0.711	0.073	0.66	0.154
F6 (n = 9)	0.822	0.007	0.593	0.093
F8 (n = 9)	0.115	0.768	0.282	0.462
All (n = 27)	0.707	< .0001	0.367	0.078

I1,016 had appeared first, it would have co-occurred with F1,534 and would have been eliminated.

Deltamethrin RR appears to be correlated with the I1,016 allele frequency but not with the C1,534 allele frequencies, whereas permethrin RR correlates with both allele frequencies (Table 7). We speculated that other resistance mechanisms are present that could drive resistance to deltamethrin, such as detoxifying enzymes or other mutations in *VGSC* that have not been identified. However, permethrin resistance appears to be driven heavily by both resistance alleles.

The I1,016 amino acid substitution has been found in many resistant populations in the Americas [18–23], and it has been shown that it is in linkage disequilibrium with C1,534 [12]. Recent work has shown that I1,016 is also in very tight disequilibrium with V410L [13].

Interestingly, the I1,016 mutation was functionally expressed in *Xenopus* oocytes and did not show an alteration in the sodium channel sensitivity to either of these pyrethroids [14]. Our data indicate a positive correlation of I1,016 allele frequency with the RR of both pyrethroids. Clearly, a greater understanding is required concerning the role that I1,016 plays in the resistance of *Ae. aegypti*. This includes the possibility that V410L [15] may be the residue to which the pyrethroid actually binds.
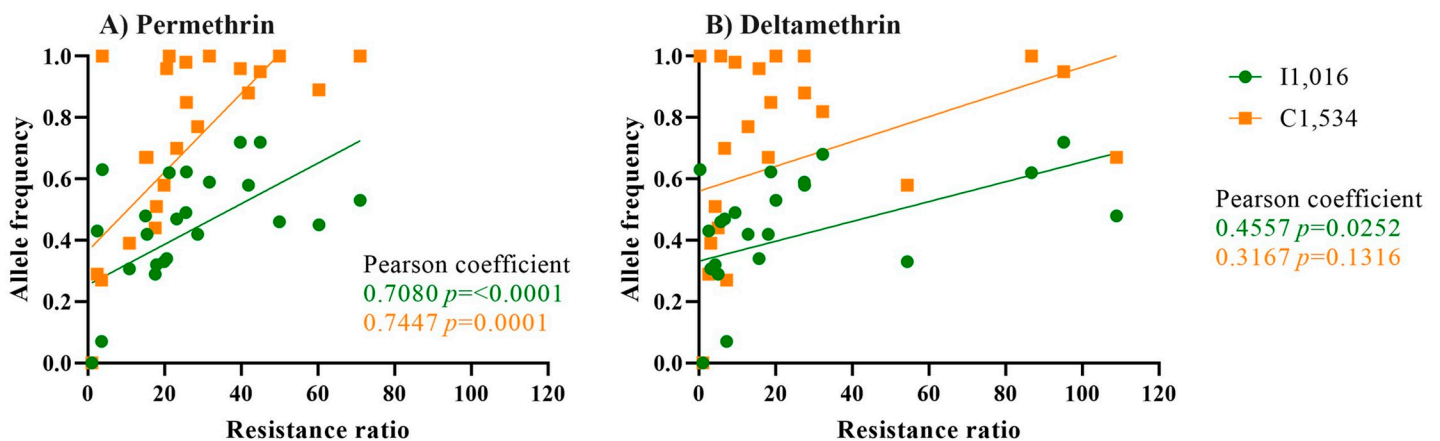
Fig 8. Correlation between the resistant alleles: I1,016 and C1,534 and the pyrethroid resistance ratio calculated for A) permethrin and B) deltamethrin.

Since many field populations are resistant to pyrethroids, and few insecticides are available on the market, negative fitness is beneficial for vector control, providing an opportunity for the use of alternative insecticides as pyrethroid resistance is lost. Pyrethroids could then be "saved" to control susceptible populations during a disease outbreak. However, the results in this study suggest that even if dominant wild-type alleles (V1,016 and F1,534) increase in a population in the absence of insecticides, recessive resistance alleles will be hidden and maintained in heterozygotes. Therefore, the initial susceptibility of populations (before the introduction of a pyrethroid) will never be recovered. Furthermore, recent work in Sao Paulo State in Brazil showed that resistance alleles persist in natural populations for at least 11 years [1].

Supporting information

S1 Table. Permethrin and deltamethrin LC50 (ug per bottle) and 95% confidence intervals calculated for eight *Aedes aegypti* colonies from Mexico. Resistance ratios (RR) were calculated at generations F3, F6 and F8 relative to the New Orleans susceptible reference strain. (DOCX)

S2 Table. Di-locus genotype counts for V1,016I and F1,534C by collection site, generation, and replicate number. VV = V1,016 homozygote, VI = V1,016I heterozygote, II = I1,016 homozygote, FF = F1,534 homozygote, FC = F1,534C heterozygote, CC = C1,534 homozygote for *Ae. aegypti* in eight generations in absence of pyrethroids. (DOCX)

S3 Table. Frequencies of resistant alleles I1,016 and C1,534 by site and generation in absence of pyrethroids. High-density intervals (HDI) 95% were calculated for each biological replicate separately. (DOCX)

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Writing – review & editing: William C. Black, IV.

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