Parallel evolution of vgsc mutations at domains IS6, IIS6 and IIIS6 in pyrethroid resistant Aedes aegypti from Mexico

Karla Saavedra-Rodriguez, Farah Vera Maloof, Corey L. Campbell, Julian Garcia-Rejon, Audrey Lenhart, Patricia Penilla, Americo Rodriguez, Arturo Acero Sandoval, Adriana E. Flores, Gustavo Ponce, Saul Lozano & William C. Black IV

Aedes aegypti is the primary urban mosquito vector of viruses causing dengue, Zika and chikungunya fevers—for which vaccines and effective pharmaceuticals are still lacking. Current strategies to suppress arbovirus outbreaks include removal of larval-breeding sites and insecticide treatment of larval and adult populations. Insecticidal control of Ae. aegypti is challenging, due to a recent rapid global increase in knockdown-resistance (kdr) to pyrethroid insecticides. Widespread, heavy use of pyrethroid space-sprays has created an immense selection pressure for kdr, which is primarily under the control of the voltage-gated sodium channel gene (vgsc). To date, eleven replacements in vgsc have been discovered, published and shown to be associated with pyrethroid resistance to varying degrees. In Mexico, F1,534C and V1,016I have co-evolved in the last 16 years across Ae. aegypti populations. Recently, a novel replacement V410L was identified in Brazil and its effect on vgsc was confirmed by electrophysiology. Herein, we screened V410L in 25 Ae. aegypti historical collections from Mexico, the first heterozygote appeared in 2002 and frequencies have increased in the last 16 years alongside V1,016I and F1,534C. Knowledge of the specific vgsc replacements and their interaction to confer resistance is essential to predict and to develop strategies for resistance management.

Pyrethroids are the most common class of insecticides used to suppress adult populations of Aedes aegypti, the principal vector of dengue, chikungunya, Zika and yellow fever viruses. The lack of vaccines for most of these arboviruses results in a tremendous reliance on chemical control. Unfortunately, intense application of pyrethroids has resulted in pyrethroid resistance in Ae. aegypti populations worldwide1–5. A major mechanism underlying pyrethroid resistance is known as knockdown resistance (kdr), which is caused by mutations in the voltage-gated sodium channel (vgsc)6. Knowledge of the specific vgsc mutations that confer resistance is essential to predict the rise of pyrethroid resistance and to develop strategies for resistance management.

Globally, eleven vgsc mutations have been identified in Ae. aegypti and in most cases, have been linked to conveying some degree of pyrethroid resistance. These include G923V, L982W, I1,011 M and V1,016 G first identified in 20037, I1,011 V and V1,016I in 20078, D1,763Y in 20099, S989P and F1,534C in 201010,11, T1,520I in 201512 and V410L in 201713. These kdr mutations are usually confined to specific geographic areas and co-occurrence of certain mutations is a common phenomenon sometimes associated with higher levels of phenotypic resistance14.

To date, only five mutations have been functionally confirmed to reduce vgsc sensitivity to pyrethroids, including S989P (IIL5–6), I1,011 M, V1,016 G (IIS6), F1,534C (IIIS6)14 and most recently V410L (IS6)13. Computer modeling predicts that pyrethroids bind to two homologous lipid exposed interfaces between domains: one

1Arthropod-borne and Infectious Disease Laboratory, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, Colorado, United States of America. 2Laboratorio de Arbovirología, Centro de Investigaciones Regionales Dr. Hideyo Noguchi, Universidad Autónoma de Yucatán, Mérida, Mexico. 3Division of Parasitic Diseases and Malaria, Centers for Disease Control and Prevention, Atlanta, Georgia, United States of America. 4Centro Regional de Investigacion en Salud Publica, Instituto Nacional de Salud Publica, Tapachula, Chiapas, Mexico. 5Laboratorio de Entomologia Medica, Facultad de Ciencias Biologicas, Universidad Autonoma de Nuevo Leon, Monterrey, Mexico. Correspondence and requests for materials should be addressed to W.C.B. (email: William.Black@colostate.edu)
binding site (PYR-1) is formed by the linker helix connecting S4 and S5 in domain II (IIL45) and domains IIS6, IIIS6, and the second binding site (PYR-2) is formed by the linker helix connecting S4 and S5 in domain I (IIA5) and domains ISS, ISS6, and IIS614–17.

In Mexico, pyrethroid-resistant \textit{Ae. aegypti} populations carry at least two \textit{vgsc} mutations, V1,016I which is linked to permethrin survival\(^8\) and F1,534C. Interestingly, F1,534C reduces permethrin binding to \textit{vgsc} channels whereas V1,016I has no effect in pyrethroid binding\(^4\). However, both replacements have co-evolved in Mexican populations; V1,016I and F1,534C allele frequencies have increased in the last 16 years\(^16\) and F1,534C has reached fixation in several locations in Southern Mexico\(^18\). The co-occurrence of V1,016I and F1,534C has been reported in several countries in Latin America, including Brazil\(^20,21\), Venezuela\(^22\), Colombia\(^23\), Martinique Island\(^24\), Puerto Rico\(^25\), Grand Cayman Islands\(^3\), Cuba\(^26\), Haiti\(^27\) and Jamaica\(^28\).

Recently, a novel mutation V410L in domain IS6 was identified in a pyrethroid resistant laboratory strain of \textit{Ae. aegypti} from Brazil\(^13\). Alone or in combination with F1,534C, V410L reduced the sensitivity of mosquito sodium channels expressed in \textit{Xenopus} oocytes to both type I (eg. permethrin) and II pyrethroids (eg. deltamethrin). Interestingly, authors did not detect this mutation in field populations from Pernambuco, Brazil, concluding the novel mutation may not yet be widespread in nature. In contrast, we identified high frequencies of V410L alongside V1,016I and F1,534C in a genome-wide association study of \textit{Ae. aegypti} from Mexico. To extend this observation, we screened the frequencies of V410L in different temporal and geographical \textit{Ae. aegypti} collections made over the last 16 years in Mexico. We compared the evolution and linkage disequilibrium of V410L alongside the V1,016I and F1,534C replacements, which occur in different domains IS6, IIS6 and IIIS6, respectively (Fig. 1). In addition, we determined the phenotype/genotype association in a field population exposed to permethrin and deltamethrin.

**Results**

**V410L in \textit{Ae. aegypti} collections from Mexico.** We determined the V410L genotypes for 1,176 mosquitoes collected from six field locations in Mexico from 2000–2016. The V410L genotype counts and calculated allele frequencies are shown in Table 1. V410L first appeared in a heterozygous individual mosquito in 2002 in Coatzacoalcos. By 2008, L410 allele frequencies ranged from 0.27–0.65 across collections. By 2012, L410 frequencies ranged from 0.56 to 0.84 in collections from the State of Veracruz. By 2014, Tapachula and Merida had allele frequencies of 0.57 and 0.9, respectively. V410L genotype frequencies were in Hardy-Weinberg equilibrium in 13 out of 14 collections where V410L was segregating. The exception was in Coatzacoalcos 2008 that had a significant deficiency of LL410 homozygotes (\(F_{IS} = 0.832\)). Figure 2 shows the allele frequencies of L410, I1,016 and C1,534 at four-time points: 2000 (\(n = 233\)), 2002–2005 (\(n = 346\)), 2006–2008 (\(n = 223\)) and 2012–2016 (\(n = 374\)) for all locations. As previously observed for I1,016 and C1,534\(^19\), L410 also increased in frequency from 2000 to 2016, noting that at each of these time points L410 and I1,016 alleles changed frequencies in parallel from 0.00 to 0.71 (Fig. 2).

Pairwise linkage disequilibrium analyses were performed between locus 410–1,016, 410–1,534 and 1,016–1,534. Table 2 shows the linkage disequilibrium coefficients (\(R_{\theta}\)) and associated probabilities obtained between pairwise loci. Twenty out of 25 collections had alleles segregating at loci 410–1,016 with \(R_{\theta}\) values ranging between 0.53–0.99 among collections; overall \(R_{\theta}\) was 0.96 (\(p = 0.0001\)). For loci 410–1,534, only five collections had mutant alleles segregating, and four were in significant linkage disequilibrium with \(R_{\theta}\) ranging from 0.33 to 0.99; the overall \(R_{\theta}\) coefficient was 0.76 (\(p = 0.0001\)). At loci 1,016–1,534, segregating alleles from six collections were in significant linkage disequilibrium, with \(R_{\theta}\) coefficients ranging from 0.31 to 0.99; overall \(R_{\theta}\) coefficient was 0.76 (\(p = 0.0001\)).

**Temporal analysis of tri-locus genotypes.** Out of 27 genotype combinations (3 genotypes at 3 loci), we found 20 genotype combinations in 1,176 individual mosquitoes collected from 2000 to 2016. Figure 3 shows the frequency of each of the 20 tri-locus genotype combinations at four-time points: 2000, 2002–2005, 2006–2008 and 2012–2016. In 2000, the triple homozygote susceptible genotype (V410/V1,016/F1,534) occurred at a high frequency (0.99) whereas a genotype including a heterozygote at loci 1,534 (V410/V1,016/F1,534) had a frequency lower than 0.01 (Fig. 3a). By 2002–2005, these genotypes were still the most common (frequencies of 0.86 and 0.08, respectively), however, six additional genotypes including homozygotes at locus 1,534 and heterozygotes at loci 1,016 and 410 occurred at very low frequencies (<0.02) (Fig. 3b). By 2006–2008, the triple
homozygote susceptible genotype (VV 410/VV 1,016/FF 1,534) and the 1,534 heterozygotes decreased to frequencies lower than 0.06. Twelve additional combinations occurred, the four most frequent genotypes were VV410/VV 1,016/CC 1,534 (frequency = 0.2), VL 410/VI 1,016/FC 1,534 (0.18), VL 410/VI 1,016/CC 1,534 and the triple resistant homozygote LL410/II 1,016/CC 1,534 (frequency = 0.18) (Fig. 3c). Observed frequencies of these genotypes were

| Site             | Year | n   | V410L genotype | L410 allele frequency and 95% HDI | FIS  |
|------------------|------|-----|----------------|-----------------------------------|------|
|                  |      |     | VV  | VL  | LL  | Freq. | lower | upper |      |
| Poza Rica        | 2000 | 46  | 0   | 0   | 0   | 0.00  | 0.003 | 0.04  |      |
|                  | 2003 | 47  | 0   | 0   | 0   | 0.00  | 0.003 | 0.04  |      |
|                  | 2008 | 39  | 17  | 17  | 65  | 0.65  | 0.5400| 0.75  | 0.04  |
|                  | 2012 | 37  | 12  | 22  | 76  | 0.76  | 0.6476| 0.84  | 0.12  |
| Martinez de la Torre | 2000 | 46  | 0   | 0   | 0   | 0.00  | 0.003 | 0.04  |      |
|                  | 2002 | 42  | 0   | 0   | 0   | 0.00  | 0.003 | 0.04  |      |
|                  | 2003 | 30  | 24  | 5   | 1   | 0.12  | 0.0586| 0.22  | 0.19  |
|                  | 2008 | 48  | 25  | 14  | 55  | 0.55  | 0.4521| 0.65  | −0.05 |
|                  | 2012 | 44  | 14  | 30  | 84  | 0.84  | 0.7555| 0.90  | −0.18 |
| Zempoala         | 2000 | 47  | 0   | 0   | 0   | 0.00  | 0.003 | 0.04  |      |
|                  | 2002 | 47  | 0   | 0   | 0   | 0.00  | 0.003 | 0.04  |      |
|                  | 2003 | 30  | 0   | 0   | 0   | 0.00  | 0.0004| 0.06  |      |
|                  | 2012 | 52  | 18  | 29  | 73  | 0.6246| 0.79  | 0.12  |      |
| Coatzacoalcos    | 2000 | 48  | 2   | 0   | 0   | 0.02  | 0.0062| 0.07  | −0.02 |
|                  | 2003 | 48  | 0   | 0   | 0   | 0.00  | 0.003 | 0.04  |      |
|                  | 2008 | 48  | 26  | 0   | 0.27 | 0.1964| 0.37  | −0.38 |
|                  | 2012 | 45  | 22  | 14  | 56  | 0.4626| 0.66  | 0.01  |      |
| Tapachula        | 2000 | 48  | 0   | 0   | 0   | 0.00  | 0.003 | 0.04  |      |
|                  | 2006 | 42  | 11  | 3   | 20  | 0.20  | 0.1307| 0.30  | 0.19  |
|                  | 2014 | 47  | 26  | 14  | 57  | 0.4732| 0.67  | −0.13 |
|                  | 2016 | 96  | 40  | 41  | 64  | 0.5653| 0.70  | 0.10  |      |
| Merida           | 2000 | 47  | 0   | 0   | 0   | 0.00  | 0.003 | 0.04  |      |
|                  | 2005 | 48  | 0   | 0   | 0   | 0.00  | 0.003 | 0.04  |      |
|                  | 2007 | 47  | 10  | 30  | 50  | 0.53  | 0.4315| 0.63  | −0.28 |
|                  | 2013 | 50  | 10  | 40  | 90  | 0.8256| 0.94  | −0.11 |
| New Orleans      | 2000 | 48  | 0   | 0   | 0   | 0.00  | 0.00  |      |      |

Table 1. V410L genotypes and allele frequencies in 25 collections from Mexico. The site, year, sample size, genotype frequency, L410 allele frequency, 95% high density intervals (HDI) and inbreeding coefficients (FIS). L = resistant allele, V = susceptible allele.

Figure 2. L410, I1,016 and C1,534 allele frequencies in 25 *Ae. aegypti* collections from Southern Mexico. Allele frequencies are plotted in four periods of time: 2000 (n = 233), 2002–2005 (n = 346), 2006–2008 (n = 223) and 2012–2016 (n = 374).

homoygote susceptible genotype (VV410/VV1,016/FF1,534) and the 1,534 heterozygotes decreased to frequencies lower than 0.06. Twelve additional combinations occurred, the four most frequent genotypes were VV410/VV1,016/CC1,534 (frequency = 0.2), VL410/VI1,016/FC1,534 (0.18), VL410/VI1,016/CC1,534 and the triple resistant homozygote LL410/II1,016/CC1,534 (frequency = 0.18) (Fig. 3c). Observed frequencies of these genotypes were
Table 2. Linkage disequilibrium coefficients (Rij), χ² and associated probabilities between loci 410-1,016, 410-1,534 and 1,016-1,534.

| Site          | Year | 410-1,016 Rij | 410-1,016 χ² | 410-1,016 P  | 1,016-1,534 Rij | 1,016-1,534 χ² | 1,016-1,534 P |
|---------------|------|--------------|-------------|-------------|----------------|---------------|-------------|
| Poza Rica     | 2008 | 0.67         | 17.3        | 0.0001      | 0.61           | 6.5           | 0.0106      |
|               | 2012 | 0.61         | 3.8         | 0.0002      | 0.31           | 3.5           | 0.0622      |
| Martinez de la Torre | 2003 | 0.53         | 8.5         | 0.0035      | 0.69           | 14.3          | 0.0002      |
|               | 2008 | 0.99         | 48.0        | 0.0001      |                |               |             |
| Zempoala      | 2012 | 0.81         | 34.5        | 0.0001      |                |               |             |
| Coatzacoalcos | 2002 | 0.99         | 50.0        | 0.0001      | 0.99           | 50.0          | 0.0001      |
|               | 2008 | 0.99         | 47.0        | 0.0001      |                |               |             |
|               | 2012 | 0.99         | 47.0        | 0.0001      |                |               |             |
| Tapachula     | 2006 | 0.99         | 42.0        | 0.0001      | 0.33           | 4.7           | 0.0303      |
|               | 2014 | 0.89         | 37.3        | 0.0001      |                |               |             |
|               | 2016 | 0.99         | 96.0        | 0.0001      |                |               |             |
| Merida        | 2007 | 0.88         | 36.0        | 0.0001      | 0.96           | 43.4          | 0.0001      |
|               | 2013 | 0.82         | 33.9        | 0.0001      |                | 0.31          | 4.8         |
| Overall population | 0.96 | 1081.7       | 0.76        | 675.9       | 0.0001         | 0.76          | 676.9       |

Significantly higher than expected (Supplementary Table S1). By 2012–2016, the triple homozygote susceptible genotype was no longer detected, and the most common genotype combinations were the triple resistant homozygote (frequency = 0.47) and VV410/Vl1,016/CC1,534 (frequency = 0.34) (Fig. 3d). Observed frequencies of these genotypes were in significant excess. In the same period, very low frequencies of resistant homozygotes at locus 410 occurred independently of 1,016 (LL410/VV1,016/CC1,534 or VV410/II1,016/CC1,534 and LL410/VV1,016/FC1,534 or VV410/II1,016/FC1,534). Also, genotypes including heterozygotes at locus 410 and homozygotes at locus1,016 and vice versa (VL410/II1,016/CC1,534 or VL410/II1,016/FC1,534 and LL410/V1,016/CC1,534 or LL410/V1,016/FC1,534) were observed at 10-fold lower frequencies (~7 individuals) than expected (Supplementary Table S1).

**Association of V410L with pyrethroid resistance.** L410 was present at a frequency of 0.69 in Viva Caucel mosquitoes used for our phenotype/genotype association study. We used a dose of permethrin or deltamethrin to discriminate three phenotypes: knockdown resistant, recovered and dead. The susceptible genotype at locus 410 was VV410, heterozygote was VL410 and resistant was LL410. Table 3 shows the outcomes of mosquitoes carrying a specific genotype in terms of response to pyrethroid treatment. For permethrin, 53% of the resistant homozygotes (LL410) were knockdown resistant, 40% recovered and only 7% died. In the heterozygote group (VL410) 4% was knockdown resistant, 28% recovered and 64% died following permethrin exposure. For deltamethrin, 72% of the resistant homozygotes (LL410) were knockdown resistant whereas 23% recovered and only 5% died. Note that the phenotype outcome is very similar between genotypes at loci 410 and 1,016. The most striking difference at locus 1,534, is that more than 92% of the heterozygotes died after exposure to permethrin or deltamethrin. Across all analyses, strong correlations were detected between phenotype and genotype.

**Association of tri-locus genotypes with pyrethroid resistance.** Because our results indicated that L410, I1,016 and C1,534 do not occur independently, we analyzed the phenotype outcome by tri-locus genotype combinations. The presence of resistant alleles in the tri-locus homozygote genotype is strongly associated with knockdown resistance and recovery for both permethrin and deltamethrin (Fig. 4a,b). Wild type genotype combinations. The presence of resistant alleles in the tri-locus homozygote genotype is strongly associated with knockdown resistance and recovery for both permethrin and deltamethrin. The double heterozygote at locus 410 and 1,016 with CC1,534 (VL410/VI1,016/CC1,534) was associated with the dead phenotype for permethrin exposure but was associated with knockdown resistance and recovery in the deltamethrin exposure group.

**Discussion**

Different replacements at residue V410 have been reported in the vgsc of several pyrethroid resistant insect species. Specifically, V410L was associated with deltamethrin resistance in the common bed bug *Cimex lectularius*19. However, replacements V410M in the tobacco budworm30 and V410A, V410G and V410M in the earworm31 have also been reported. In *Ae. aegypti*, V410L was recently detected in a pyrethroid resistant insectary strain from Brazil33, which demonstrated that V410L alone or in combination with the F1,534C reduced the sensitivity of mosquito sodium channels expressed in *Xenopus* oocytes to both type I and II pyrethroids. In the same study, V410L was not detected in a small field survey in the State of Pernambuco, Brazil, and authors suggested that V410L was not yet widespread in the field. Importantly, our results show that V410L has existed for at least 16 years in Mexico, the first heterozygote was detected in 2002 in Coatzacoalcos, Veracruz, and has increased gradually to high frequencies in 2016 (up to 0.92). Interestingly, we found L410 to be in greater linkage disequilibrium
with I1,016 than with C1,534. Our previous study measured linkage disequilibrium between I1,016 and C1,534 and we proposed a sequential model, wherein C1,534 first occurred (providing low levels of resistance) and then the replacement I1,016 occurred with this haplotype, providing even higher levels of resistance. V410L challenges this sequential model, in which both V410L and V1,016I might have occurred independently on a C1,534 haplotype and then became \( cis \) to C1,534 by recombination. An alternative model assumes the three mutations arose independently at very low frequencies, and then by two recombination events, came to occur in a \( cis \) arrangement. Our results indicate that C1,534 was at a frequency of 0.004 in collections from 2000 whereas L410 and I1,016 were below limits of detection. By 2002–2005, C1,534 was at higher frequency (0.055), while L410 and I1,016 appeared at lower frequencies (0.013 and 0.02, respectively). During this period, we identified heterozygotes at 410 and 1,016 occurring independently in a FF1,534 background. Also, the triple heterozygote was identified in low frequencies (5/346). By 2006–2008 heterozygotes VL 410 and VI 1,016 on a FC 1,534 heterozygote or CC 1,534 homozygote were favored by selection, whilst mutant genotypes at 410 and 1,016 never occurred independently or were at very low frequencies.

A recent study found that V410L and F1,534C occurred without V1,016I in a deltamethrin resistant laboratory strain originated from Rio de Janeiro. In contrast, we show large linkage disequilibrium between L410 and I1,016, except among very few individuals collected early in 2002–2005; however, this genotype combination was no longer detected in following years. Whether L410 remains independent of I1,016 in Brazil will provide evidence of the mutations arising independently at a local level in the sequential model. However, I1,016 and C1,534 are already widespread in several regions of Brazil, and due to high migration rates among \textit{Ae. aegypti}

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**Figure 3.** Frequencies of the 20 tri-locus genotypes plotted by periods of time. (a) Frequencies in 2000. (b) Frequencies in 2002–2005. (c) Frequencies in 2006–2008 and (d) Frequencies in 2012–2016. The order of the genotypes is 410/1,016/1,534. Resistant allele at 410 = L, 1,016 = I and 1,534 = C. The triple susceptible genotype is at the bottom of each graph whereas the triple resistant genotype is shown at the top of each graph.
In populations, we would expect I1,016 and C1,534 to recombine with L410-C1,534 in future years. However, an alternative scenario is that as in Mexico, L410 is already present at high frequencies in Brazilian collections previously genotyped with I1,016-C1,534 but simply has not yet been detected.

The selection of the triple homozygote resistant genotypes detected in our data suggests higher fitness of this genotype in the presence of pyrethroids. The role of L410 and C1,534 in conferring pyrethroid resistance was determined in Haddi et al. 2016. L410 alone or in combination with C1,534 confers high levels of resistance.

### Table 3.

| Genotype | Permethrin | Deltamethrin |
|----------|------------|--------------|
|          | Knockdown resistant | Recovered | Dead | Knockdown resistant | Recovered | Dead |
| n 94    | n 95        | n 95        |       | n 111        | n 67       | n 92  |
| V410L LL | 87 (53%)    | 66 (40%)    | 12 (7%)| 86 (72%)    | 28 (23%)   | 6 (5%) |
|          | 4.0 × 10^{-30} |          |       | 6.1 × 10^{-32} |          |       |
|          | V1,016I II  | 90 (54%)    | 66 (40%)| 11 (7%)     | 86 (70%)   | 27 (22%)| 9 (7%) |
|          | 3 (3%)      | 28 (32%)    | 57 (65%)| 24 (20%)    | 36 (30%)   | 52 (46%)|
|          | 1 (3%)      | 1 (3%)      | 27 (93%)| 1 (1%)      | 4 (3%)     | 31 (86%)|
|          | 1.4 × 10^{-32} |          |       | 1.2 × 10^{-33} |          |       |
| F1,534C CC | 90 (37%)    | 95 (39%)    | 58 (24%)| 111 (56%)   | 65 (33%)   | 24 (12%)|
|          | 3 (8%)      | 0 (0%)      | 36 (92%)| 0 (0%)      | 2 (1%)     | 61 (97%)|
|          | 1 (50%)     | 0 (0%)      | 1 (50%) | 0 (0%)      | 0 (0%)     | 6 (100%)|
|          | 7.9 × 10^{-15} |          |       | 6.8 × 10^{-13} |          |       |

Figure 4. Frequencies of tri-locus genotypes in knockdown resistant, recovered and dead mosquitoes following (a) permethrin or (b) deltamethrin exposure. The order of the genotypes is shown for locus 410/1,016/1,534 respectively. Resistant allele at loci 410 = L, 1,016 = I and 1,534 = C. The triple susceptible genotype is closer to the y axis whereas the triple resistant genotype is shown on the far right side of the x axis.
however, it remains to be seen if it is fit in the field. We found 4 out of 1,176 individuals with L410 and C1,534 occurring independently (in the absence of 1,016), and this genotype became extinct in Mexican populations. In our phenotype and genotype studies, the triple homozygote resistant individuals had better survival (knockdown resistance and recovery) following either permethrin or deltamethrin exposure. One particular genotype, VL410/VI1,016/CC1,534, had different outcome depending of the specific pyrethroid, with this genotype associated with dead in mosquitoes following permethrin exposure. In contrast, this genotype was mostly associated with survival (knockdown and recovery) in mosquitoes exposed to deltamethrin. Apparently, the presence of heterozygotes at loci 410 and 1,016 was sufficient for deltamethrin survival.

F1,534C is located in the PYR-1 receptor site and is responsible for reducing vgsc sensitivity to permethrin. Although residue 1,016 is located in the PYR-1 receptor site, only the V1,016G replacement occurring in Aedes aegypti from Asia reduces vgsc whilst the V1,016f replacement found in the Americas does not16. In contrast, V410L is located in DIS6 but does not form part of the PYR-2 receptor site. It has been suggested that the reduction of sodium channel sensitivity to permethrin and deltamethrin by V410L might result from changes in the gating properties of vgsc without inhibiting molecule docking13. Because pyrethroids prefer to bind to sodium channels in the open state, kdr mutations that deter the open state would counteract the pyrethroid effects13. In recent structural models, pyrethroids make multiple contacts with helices III45, IS5, IS6, and IS6e as well as II45, IS5, IS6, and IS6e that would maintain vgsc in an open state17-23. Simultaneous binding of pyrethroids to both PYR-1 and PYR-2 is thought to effectively prolong the opening of vgsc24. It is possible that co-occurrence of V410L and V1,016f, although in different receptor sites, provide fitness advantages in the presence of pyrethroids, thus favoring co-selection. The interaction of both mutations in electrophysiology experiments will address if the co-occurrence of these mutations is compensatory or synergistic in the presence of pyrethroids.

Methods

Detection of V410L and genotyping. Primer positions at exon 9 and 10 of vgsc in Aedes aegypti are shown in Supplementary Figure 1. We used primers 410fw 5′-GATAATCCAAATCAGGGTGATA-3′ and 410rev 5′-TTCTTCTCAGCGCCCTCTT-3′ to amplify a ~500 bp region that flanked exon 9, intron 9–10 (~239 bp) and exon 10. PCR reactions were performed using 12.5 µl of GoGreenTag (Promega, Madison WI), 11.65 µl H2O, 1.1 µl of each primer (at 50 pmol/µl) and 1 µl genomic DNA (~25 ng). PCR conditions were 3 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 60°C and 1 min at 72°C and a final extension of 5 min at 72°C. Products were purified using the MinElute PCR purification columns (Qiagen, Hilden Germany) and sequenced using the primers 410_ex9fw 5′-TAGCATACGTGGACCGTTG and 410rev targeting a fragment of 174 bp in exon 10. Sequences were analyzed using the Geneious software (Biomatters Inc, Newark NJ).

Following identification of the G (GTA = Val) and T (TTA = Leu) alleles at locus 410, we designed an allele-specific PCR system to detect individual genotypes by melting curve analysis6. Each reaction contained 50 µM of each forward primer V410fw (5′-GGGGGAGGGCGGCCGAGGCGTACCTTTCTGGTTTGTTCTACGTG-3′), and L410fw (5′-GGGGGAGGGCGCCGTCCTATTGTTGTTCTACGTG-3′) and 100 µM of reverse primer 410rev (5′-TTCTTCTCAGCGCCCTCTT-3′), 10 µl Sybr Green Master (BioRad, Hercules CA), 9.7 µl ddH2O and 1 µl of genomic DNA (~25 ng). PCR and melting curve analysis was run in a CFX-96 (BioRad) following 3 min at 95°C, 39 cycles of 10 sec at 95°C, 10 sec at 60°C, 30 sec at 72°C followed by a melting curve from 65°C to 95°C with increments of 0.2°C during 10 sec. The products consisted of a single 133 bp amplicon for a VV 410 homozygote, a single 113 bp amplicon for a L410 homozygote (resistant) and the presence of both amplicons in heterozygote individuals (VL410) (Supplementary Figure 2).

Mosquito collections. We determined the V410L genotypes for 1,176 mosquitoes collected from six field locations in Mexico from 2000–2016 (Supplementary Table S2 and Supplementary Figure 3). Four of these sites were in the State of Veracruz (Eastern Mexico). Tapachula is in the State of Chiapas in Southwestern Mexico and borders Guatemala, while Merida is in the Yucatan peninsula in Southeastern Mexico. The V410L database was completed with genotypes at loci 1,016 and 1,53419,25.

Allele frequencies and linkage disequilibrium analysis. V410L allele frequencies were calculated from genotypic frequencies following Garcia et al.18. The 95% high density intervals (HDI 95%) around these frequencies were calculated in WINbugs2.0 following 1,000,000 iterations. Departure from Hardy-Weinberg expectations were expressed as Wright’s inbreeding coefficient (FIS) and a χ2 test was used to test the hypothesis FIS = 0 (d.f. = 1). Pairwise linkage disequilibrium between alleles at loci 410 and 1,016 or between loci 410 and 1,534 was calculated using LINKD2S following Vera-Maloof et al.19. Composite disequilibrium between resistant alleles was tested and a χ2 test determined if significant disequilibrium existed among alleles at both loci.

V410L association with pyrethroid resistance. To determine phenotype/genotype associations, we used the Viva Caucel (long – 89.71827, lat 20.99827) collection from Yucatan, Mexico made in 2011. First, we calculated the permethrin and deltamethrin (Chem Service) lethal concentration that killed 50% of the mosquitoes (LC50) in 3–4 days old adults of the F1 generation. The insecticide treatment consisted of a 1 h exposure in a insecticide coated bottle, transfer of mosquitoes to recovery chambers and mortality scored at 24 h after treatment. We assessed the levels of permethrin and deltamethrin resistance in Viva Caucel relative to the New Orleans (NO) reference strain. The permethrin LC50 was 47.9-fold higher than NO (26.5 µg vs 0.55 µg, respectively) and the deltamethrin LC50 was 47.6-fold higher than NO (10.49 µg vs 0.22 µg).

Once the LC50 was calculated, six to 10 groups of 50 mosquitoes at a time were exposed to 25 µg of permethrin or 3 µg of deltamethrin coated in 250 µL Wheaton bottles during 1 h. Immediately following exposure, active (knockdown resistant) and inactive mosquitoes were transferred to separate containers. To ensure correct categorization, we phenotyped mosquitoes 4 h after treatment. We observed activity and if the mosquitoes were capable
of flight, they were scored as ‘knockdown resistant’. In the inactive group we separated the newly recovered mosquitoes from the inactive mosquitoes and scored them as ‘recovered’ and ‘dead’. Supplementary Table 3 shows the total number of mosquitoes exposed to permethrin and deltamethrin and the distribution between the three phenotypic categories.

A subsample of mosquitoes from each group was individually frozen; DNA was isolated by the salt extraction method and resuspended in 150 μl of TE buffer (10 mM Tris–HCl, 1 mM EDTA pH 8.0). For the Viva Cauzel mosquitoes exposed to permethrin we randomly selected 95 knockdown-resistant, 95 recovered and 95 dead mosquitoes. For deltamethrin we randomly selected 111 knockdown-resistant, 67 recovered and 92 dead mosquitoes. We conducted genotyping at locus 410 using the V410L melting curve system described above. For V1,016I and F1,534C genotypes, we used previously described methodologies. A contingency table was used to test for association between the phenotypes (knockdown resistant, recovered and dead) and genotypes (mutant homozygote, wild type homozygote, and heterozygote) at each locus separately (410, 1,016 and 1,534) and for the 27 (3 × 3 × 3) tri-locus genotype combinations.

Disclaimer. The findings and conclusions in this paper are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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**Author Contributions**

K.S. and W.C.B. conceived/designed the research. K.S., F.V. and A.A. conducted the experiments. A.L., J.G., P.P., A.R., A.E.F., S.L. and G.P. contributed mosquito colonies/reagents/or analytical tools. C.L.C., F.V., S.L., K.S. and W.C.B. analyzed the data. K.S., F.V. and W.C.B. wrote the manuscript. All authors read, corrected and approved the manuscript.

**Additional Information**

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