Mechanisms associated with pyrethroid resistance in populations of *Aedes aegypti* (Diptera: Culicidae) from the Caribbean coast of Colombia

Paula X. Pareja-Loaiza, Liliana Santacoloma Varon, Gabriela Rey Vega, Doris Gómez-Camargo, Ronald Maestre-Serrano, Audrey Lenhart

1 Estudiante, Doctorados Nacionales Colciencias, Grupo UNIMOL, Doctorado en Medicina Tropical, Facultad de Medicina, Universidad de Cartagena, Cartagena de Indias, Colombia. 2 Laboratorio de Entomología, Subdirección Laboratorio Nacional de Referencia, Dirección Redes en Salud Pública, Instituto Nacional de Salud, Bogotá, Colombia. 3 Grupo UNIMOL, Doctorado en Medicina Tropical, Facultad de Medicina, Universidad de Cartagena, Cartagena de Indias, Colombia. 4 Facultad de Ciencias de la Salud, Universidad Simón Bolívar, Barranquilla, Colombia. 5 Centers for Disease Control and Prevention, Atlanta, Georgia, United States of America

*paxipa82@gmail.com*

**Abstract**

*Aedes aegypti* is the main vector of dengue, chikungunya, and Zika viruses, which are of great public health importance in Colombia. *Aedes* control strategies in Colombia rely heavily on the use of organophosphate and pyrethroid insecticides, providing constant selection pressure and the emergence of resistant populations. In recent years, insecticide use has increased due to the increased incidence of dengue and recent introductions of chikungunya and Zika. In the present study, pyrethroid resistance was studied across six populations of *Ae. aegypti* from the Caribbean coast of Colombia. Susceptibility to **λ**-cyhalothrin, deltamethrin, and permethrin was assessed, and resistance intensity was determined. Activity levels of enzymes associated with resistance were measured, and the frequencies of three *kdr* alleles (V1016I, F1534C, V410L) were calculated. Results showed variations in pyrethroid susceptibility across *Ae. aegypti* populations and altered enzyme activity levels were detected. The *kdr* alleles were detected in all populations, with high variations in frequencies: V1016I (frequency ranging from 0.15–0.70), F1534C (range 0.94–1.00), and V410L (range 0.05–0.72). In assays of phenotyped individuals, associations were observed between the presence of V1016I, F1534C, and V410L alleles and resistance to the evaluated pyrethroids, as well as between the V1016I/CC1534/VL410 tri-locus genotype and **λ**-cyhalothrin and permethrin resistance. The results of the present study contribute to the knowledge of the mechanisms underlying the resistance to key pyrethroids used to control *Ae. aegypti* along the Caribbean coast of Colombia.
Introduction

*Aedes aegypti* (*Stegomyia aegypti*) (Linnaeus, 1762) is the main vector of the dengue (DENV), chikungunya (CHIKV), and Zika (ZIKV) viruses. The diseases caused by these viruses are of growing public health importance worldwide owing to increased proliferation of mosquito populations, increased urbanization, as well as climatic and other environmental conditions suitable for transmission [1].

Globally, the burden of disease caused by dengue is increasing; it is estimated that approximately 390 million dengue infections occur each year, of which 96 million manifest clinically [2]. In 2015, 2.35 million cases of dengue were reported in the Americas, of which >10,200 cases were diagnosed as severe dengue, causing 1,181 deaths. In Colombia, dengue is considered a public health priority owing to its endemic transmission as well as the increased occurrence of severe dengue outbreaks, simultaneous circulation of all four DENV serotypes, and the occurrence of epidemic cycles every 2–3 years. In Colombia, the largest dengue epidemic was recorded in 2010, with >150,000 confirmed cases and 217 deaths [3]. Moreover, during 2007–2017, 609,228 cases of dengue were reported, of which 119,888 (19.7%) occurred in the Caribbean Region, specifically in the departments of Atlántico, Cesar, Córdoba, Sucre, Bolívar, La Guajira, Magdalena, and San Andrés y Providencia [4].

Chikungunya and Zika viruses were recently introduced into Colombia, in 2014 and 2015, respectively [5, 6]. The prevention and control activities of these three *Aedes*-borne arboviruses in Colombia have focused mainly on their principal vector, *Ae. aegypti*, through educational campaigns aimed at the community for the elimination of breeding sites, the application of biological and chemical insecticides, and the use of growth [7, 8]. The constant selection pressure arising from the heavy use of pyrethroid and organophosphate insecticides has generated resistant populations of *Ae. aegypti* in multiple areas of Colombia [9–15].

Resistance to insecticides in mosquitoes can be caused by the following mechanisms: behavioral modifications resulting in lessened likelihood of exposure, decreased penetration of the insecticide across the mosquito cuticle, alterations occurring at the insecticide target site within the mosquito, and increased detoxification (also referred to as metabolic resistance); the latter two mechanisms are the most frequently studied [16]. Target site alterations are most commonly caused by *kdr* mutations on the voltage-dependent sodium channel gene *para*, which is the target site for pyrethroids and DDT, or by mutations on the *Ace-1* gene (coding for the enzyme acetylcholinesterase), which is the target site for organophosphate and carbamate insecticides [17]. Metabolic resistance arises due to the increased activity or expression of genes coding for the main detoxifying enzymes including glutathione S-transferases, mixed-function oxidases, and esterases [16].

In Colombia, the insecticide susceptibility status of *Ae. aegypti* populations has been monitored for more than a decade. Since 2004, the National Insecticide Resistance Surveillance Network, headed by Colombia’s National Institute of Health, has evaluated approximately 170 populations of *Ae. aegypti* in 26 of the 32 departments in Colombia. The findings demonstrate variability in susceptibility to the insecticides temephos, λ-cyhalothrin, deltamethrin, permethrin, cyfluthrin, etofenprox, malathion, fenitrothion, pirimiphos-methyl, bendiocarb, and propoxur [12–29]. Moreover, increased activity levels of insecticide-degrading enzymes, such as nonspecific esterases, mixed-function oxidases (MFOs), glutathione S-transferases (GSTs), and insensitive acetylcholinesterase (iAChE), have been observed in resistant populations [9–13, 24]. In addition, the *kdr* mutations V1016I [13, 30], F1534C [31], and V410L [15] associated with pyrethroid resistance have recently been detected.

Specifically, in the Caribbean region, populations of *Ae. aegypti* have been reported with variable levels of susceptibility to organophosphates, resistance to pyrethroids, and alterations
in the enzymatic activity of glutathione-S-transferases, alpha-estrases and mixed function oxidases [13]. Additionally, the kdr mutations V1016I [13, 30], F1534C [31, 32] and V410L [15] have been detected.

The present study builds upon earlier work by further investigating the intensity and spatial extent of pyrethroid resistance in *Ae. aegypti* along the Caribbean coast of Colombia and links the frequency of kdr alleles and tri-locus kdr haplotypes to insecticide resistant phenotypes. To further understand the mechanisms of resistance, we also analyzed the activity levels of key detoxification enzyme groups.

**Materials and methods**

The insectary that was used to rear mosquitoes belonged to the Public Health Laboratory of the Department of Atlántico. Mosquitoes were reared using their existing protocols which include the routine use of feeding mosquito colonies on mice. This is standard practice in government insectaries in Colombia and is overseen by the Colombian National Institute of Health’s National Public Health Laboratory Network.

*Ae. aegypti* collections

*Ae. aegypti* were collected in the municipalities of Barranquilla (N 10° 57’ 10.622”, W 75° 49’ 12.024”) and Juan de Acosta (N 10° 49’ 44.731”, W 75° 2’ 9.088”) in the department of Atlántico; Cartagena (N 10° 24’ 55.416”, W 75° 27’ 38.485”) in the department of Bolívar; Valledupar (N 9° 56’ 55.068”, W 73° 38’ 4.164”) and Chiriguaná (N 9° 21’ 41.27”, W 73° 35’ 58.919”) in the department of Cesar; and Montería (N 8° 44’ 30.866”, W 75° 52’ 0.433”) in the department of Córdoba (Fig 1).

The study was undertaken in collaboration with and under the supervision of the Entomology Laboratory of the Colombian National Institute of Health, which coordinates the

**Fig 1. Aedes aegypti collection sites located in the Caribbean region of Colombia.** Montería (red), Bolivar (yellow), Atlántico (blue) and Cesar (orange).

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Entomology Laboratory Network in Colombia. This network for entomological surveillance includes the monitoring of insecticide resistance in insects of public health importance, to guide decision-making in the control of vector-borne diseases at the national level. The project also had the support of the local health secretaries in each of the departments selected for sampling sites.

The study sites were selected taking into account the national insecticide susceptibility baseline records. Municipalities with a susceptibility baseline were selected in order to identify variations in susceptibility over time and expand knowledge regarding enzymatic and molecular resistance mechanisms. Sites without an existing baseline were also selected in order to generate baseline data regarding the susceptibility to insecticides in these areas.

Immature *Aedes* were collected from habitats including tanks, pools, plastic/metallic cans, tires, animal water dishes, and flower vases located around houses. The specimens were reared to adults and maintained under controlled conditions of temperature (28°C ± 2°C), relative humidity (60% ± 10%), and photoperiod (12 h light:12 h dark) in the Public Health Laboratory of the department of Atlántico.

Upon emergence, male mosquitoes were fed with 10% sugar solution, and the females were fed with mouse (*Mus musculus*) blood every third day to obtain eggs of the F1 generation. Eggs were stored in an airtight plastic container, until they were hatched to obtain the adult mosquitoes used in the bioassays.

**Bioassays**

Insecticide bioassays were performed following the methodologies described by the CDC [33] and WHO [34]. The pyrethroid insecticides and their concentrations were as follows: λ-cyhalothrin (10 μg/bottle [CDC] and 0.03% treated papers WHO), deltamethrin (10 μg/bottle [CDC] and 0.03% treated papers WHO), and permethrin (15 μg/bottle [CDC] and 0.25% treated papers WHO). The technical grade insecticides (Chem Service®) used for the CDC bioassays were provided by the National Insecticide Resistance Surveillance Network of the Colombian National Institute of Health. The insecticide-impregnated papers used for the WHO bioassays were provided by University Sains Malaysia.

For each population, 20–25 F1 generation, 3- to 5-day-old, unfed female *Ae. aegypti* were used in each bioassay replicate; as a control, the susceptible Rockefeller laboratory *Ae. aegypti* strain was used. Each bioassay consisted of four replicates per insecticide for each population. The diagnostic time post-exposure was 30 min for the CDC bioassays and 24h for the WHO bioassays. Upon the completion of the diagnostic time, the living and dead specimens were classified as phenotypically resistant (R) or susceptible (S), and individually stored in 0.5 mL tubes with a hole in the lid and desiccated in tightly sealed bags containing silica gel. The bags containing the tubes were stored at −80°C for the subsequent detection of the V1016I, F1534C, and V410L *kdr* alleles.

In populations where resistance was detected via the CDC bioassay, resistance intensity was determined by conducting additional bioassays employing 2X the original insecticide concentration [34].

**Biochemical assays**

Biochemical assays were conducted on F1 generation adults. One day post-emergence, 40 unfed female *Ae. aegypti* from each population were preserved at −80°C until processing. Individuals from the susceptible Rockefeller strain were used as controls. Mosquitoes were homogenized individually in 30 μL of distilled water for 5–10 seconds with an electric macerator and an additional 270 μL of distilled water was added for a final volume of 300 μL. Subsequently,
samples were centrifuged at 12,000 rpm for 60 seconds and aliquoted in triplicate in 96 well microplates: 10 μL for α, β, pNPA-esterases; 15 μL for GST; 20 μL for mixed-function oxidases (MFO); and 25 μL for iAChE. For the tests of MFO and acetylcholinesterase, the samples were transferred without being centrifuged. Enzyme activity levels were determined according to the methodology described by Valle et al. [35], which measures the optical densities at predetermined wavelengths to estimate the activity levels of MFO, iAChE, esterases, and GSTs. Total protein concentration was also determined for each individual mosquito to correct for differences in body sizes [36]. Results were read using an ELISA plate reader (Multiskan™ - Thermo Fisher Scientific).

Detection of kdr alleles
Real-time PCR was used to identify the V1016I, F1534C, and V410L kdr mutations. To estimate the allele frequencies in natural populations, 40–50 Ae. aegypti parental (F0) mosquitoes from each population were analyzed. To estimate associations between genotype and phenotype, all phenotypically resistant (R) and 30 randomly selected susceptible (S) individuals were analyzed per insecticide per population.

DNA was extracted from individual mosquitoes using the Quanta Biosciences Extracta™ Kit. Individual mosquitoes were placed in sterile 0.2 mL tubes and 25 μL extraction buffer was added to each tube, followed by an incubation at 95°C for 30 min in a C1000 Bio-Rad CFX 96 Touch™ Real-Time System thermocycler. At the end of the incubation, 25 μL of stabilization buffer was added. DNA was quantified using a NanoDrop™ 2000/2000c spectrophotometer (ThermoFisher Scientific).

PCR reactions were performed in a Bio-Rad C1000 CFX96 Real-Time System thermocycler. Genotype was determined by analyzing the melting curves of the PCR products. The V1016I mutation was amplified following the methodology described by Saavedra-Rodrıíguez et al. [37], using a final reaction volume of 20 μL, containing 6 μL of ddH₂O, 10 μL of iQ™ SYBR® Green Supermix (Bio-Rad), 1 μL of each of the V1016f, I1016f, and I1016r primers (Table 1), and 1 μL of DNA template. The cycling conditions were as follows: an initial denaturation at 95°C for 3 min followed by 40 cycles of: 95°C for 10 s, 60°C for 10 s, and 72°C for 30 s; and a final extension at 95°C for 10 s. The melting curves were determined by a denaturation gradient from 65°C to 95°C with an increase of 0.2°C every 10 seconds.

The F1534C mutation was detected following the methodology described by Yanola et al. [38], using a final reaction volume of 20 μL comprised of 7.15 μL of ddH₂O, 9 μL of iQ™ SYBR® Green Supermix (Bio-Rad), 0.6 μL of each of the F1534f, C1534f, and F1534r primers (Table 1), 0.65 μL of the C1534f primers, and 2 μL of DNA template. The cycling conditions were as follows: an initial denaturation at 95°C for 3 min followed by 37 cycles of: 95°C for

Table 1. Primer sequences used for detecting kdr alleles.

| Mutation | Primer | Sequence (5´–3´) |
|----------|--------|-----------------|
| V1016I   | V1016(f) | 5´-CCGGCAAGGCGCCGCCGCCGCCGCCACAAATGGTTTCCCACCCGCCACC-3´ |
|          | I1016(f) | 5´-GGGAGCACTTCTTTCCAGGGCAGGGGC-3´ |
|          | I1016(r) | 5´-GGATGAAAGGCAATTTGGACAAAGAC-3´ |
| F1534C   | C1534(f) | 5´-GGGGGCAAGGCGCCGCCGCCGCCGCCACTTCTTTCCAGGGCAGGGGC-3´ |
|          | F1534(f) | 5´-GGGGTCTTCTTTCTTTCTCTCTTCTCATATT-3´ |
|          | F1534(r) | 5´-TCCTGCTGCGTCAAATCTCCAGGATTCG-3´ |
| V410L    | V410(f)  | 5´-GGGGCAAGGGGCGCCGCCGCCGCCGCCATCTTCTTTCCAGGGCAGGGGC-3´ |
|          | L410(f)  | 5´-GGGGCATCTTTCTCTTTCTTTCTCTCTTCCATATT-3´ |
|          | L410(r)  | 5´-TTCTTCTGGCCGGCCCTCTTT-3´ |

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10 s, 57˚C for 30 s, and 72˚C for 30 s; and a final extension at 95˚C for 10 s. The melting curves were determined by a denaturation gradient from 65˚C to 95˚C with an increase of 0.5˚C every 5 s.

The V410L mutation was detected following the methodology described by Haddi et al. [39], using a final reaction volume of 21 μL comprised of 9.6 μL of ddH₂O, 10 μL of iQ™ SYBR® Green Supermix (Bio-Rad), 0.1 μL of each of the L410f, V410f, and L410r primers (Table 1), 0.2 μL of the L410r primer, and 1.0 μL of DNA template. The cycling conditions were as follows: an initial denaturation at 95˚C for 3 min followed by 39 cycles of: 95˚C for 10 s, 60˚C for 10 s, and 72˚C for 30 s; and a final extension at 95˚C for 10 s. The melting curves were determined by a denaturation gradient from 65˚C to 95˚C with an increase of 0.2˚C every 10 s.

Each mosquito was analyzed in duplicate. For all assays for each mutation, three positive controls were included: a wild-type homozygote, a homozygote mutant, and a heterozygote. All assays also included a negative control consisting of master mix without DNA template.

Data analysis

**Bioassays.** Mortality was scored at the diagnostic time per insecticide per population. Populations were categorized according to the WHO criteria [34], whereby 98%–100% mortality indicates susceptibility, 90%–97% suggests resistance is developing and <90% mortality indicates resistance.

**Biochemical assays.** Absorbance values were entered into Excel databases to calculate the mean and standard deviation for each mosquito. To express the absorbance values in terms of enzymatic activity, data regarding the homogenate volume of each mosquito, total protein content for each mosquito, and units of activity for each enzyme group were calculated according to the protocol described by Valle et al. [35]. The cutoff value for the susceptible Rockefeller strain was determined based on the 99th percentile of absorbance, and the percentage of individuals from the field strains with activity levels that exceeded this cutoff value were classified according to the criteria proposed by Montella et al. [40]: <15% unaltered, 15%–50% altered, and >50% highly altered.

After determining the activity levels for each enzyme group, an analysis of variance was performed, followed by Tukey’s multiple comparison test, with the significance level set at \( p \leq 0.05 \), to identify populations with any statistically significant differences as compared to the Rockefeller reference strain.

**Allelic and genotypic frequencies of the V1016I, F1534C, and V410L mutations.** Results were obtained using Bio-Rad’s Precision Melt Analysis Software™ and were interpreted as follows. For the V1016I mutation, a melting peak at 77˚C corresponded to a mutant homozygote (I/I), a peak at 82˚C corresponded to a wild-type homozygote (V/V), and peaks at both 77˚C and 82˚C corresponded to a heterozygote (V/I). For the F1534C mutation, a peak at 82˚C corresponded to a mutant homozygote (C/C), a peak at 78˚C corresponded to a wild-type homozygote (F/F), and peaks at both 78˚C and 82˚C corresponded to a heterozygote (F/C). For the V410L mutation, a peak at 80˚C corresponded to a mutant homozygote (L/L), a peak at 83˚C corresponded to a wild-type homozygote (V/V), and peaks at both 80˚C and 83˚C corresponded to a heterozygote (V/L).

From the parental mosquitoes (F0), the population-level allele frequencies for I1016, C1534, and L410 were calculated using Eq (1) as follows

\[
\frac{n \text{ heterozygotes} + 2 \cdot n \text{ homozygotes}}{2 \cdot (\text{total n mosquitoes analyzed})}
\]  

(1)
The genotypic frequencies for $V_{1016}/V_{1016}$, $F_{1534}/F_{1534}$, $V_{410}/V_{410}$, $I_{1016}/I_{1016}$, $C_{1534}/C_{1534}$, $L_{410}/L_{410}$, $V_{1016}/I_{1016}$, $F_{1534}/C_{1534}$, $V_{410}/L_{410}$ were calculated using Eq (2).

\[
\text{The Hardy–Weinberg principle was tested, as shown in Eq (3)}
\]

\[
p^2 + 2pq + q^2 = 1
\]

where $p$ is the number of wild-type homozygotes, $pq$ is the frequency of heterozygotes, and $q$ is the frequency of mutant homozygotes.

Expected wild-type $V_{1016}/V_{1016}$, $F_{1534}/F_{1534}$, $V_{410}/V_{410}$ homozygotes = $p^2$ (n).

Expected $V_{1016}/I_{1016}$, $F_{1534}/C_{1534}$, $V_{410}/L_{410}$ heterozygotes = $2pq$ (n).

Expected mutant $I_{1016}/I_{1016}$, $C_{1534}/C_{1534}$, $L_{410}/L_{410}$ homozygotes = $q^2$ (n).

The Chi square test was used to determine whether the populations were in Hardy–Weinberg equilibrium, as shown in Eq (4):

\[
x^2_{\text{calc}} = \sum \left( \frac{f_0 - f_e}{f_e} \right)^2
\]

$f_0$: Frequency observed value.

$f_e$: Frequency expected value.

If the calculated value of $\chi^2$ was < tabulated $\chi^2$ (1 gl) = 3.84 and $P < 0.05$, the $H_0$ that the study population was in Hardy–Weinberg equilibrium was accepted; otherwise, if the calculated $\chi^2$ was $\geq$ tabulated $\chi^2$, the $H_a$ that the study population was not in Hardy–Weinberg equilibrium was accepted.

In addition, the coefficient of endogamy was calculated using Eq (5) as follows:

\[
F_{IS} = 1 - \left( \frac{H_{\text{obs}}}{H_{\text{exp}}} \right)
\]

where, $H_{\text{obs}}$ is the number of observed heterozygotes and $H_{\text{exp}}$ is the number of expected heterozygotes; if $F_{IS}$ was significantly higher than 0, an excess of homozygotes was considered, and if $F_{IS}$ was significantly less than 0, an excess of heterozygotes was considered in the population, with a significance of $P < 0.05$. In addition, the frequencies of tri-locus genotypes were determined in the study populations.

**Association of kdr mutations with pyrethroid resistance.** The association between resistant and susceptible phenotypes and their kdr genotypes was tested using contingency tables, and the relationship between phenotype and tri-locus genotype was tested using the statistical software programs OpenEpi version 3.0 (https://www.openepi.com/TwobyTwo/TwobyTwo.htm) and GraphPad Prism version 8.1.

**Results**

**Bioassays**

A total of 1732 adult female *Ae. aegypti* were tested in WHO bioassays for susceptibility to λ-cyhalothrin ($n = 564$), deltamethrin ($n = 586$), and permethrin ($n = 582$). Resistance to λ-cyhalothrin and permethrin was detected in all six evaluated populations. Resistance was most frequent in Montería with 43.3% mortality to λ-cyhalothrin and 24.0% mortality to permethrin. Cartagena was the least resistant, with mortalities of 86.4% to λ-cyhalothrin and 77.6% to permethrin. Susceptibility to deltamethrin was observed in the populations from Juan de Acosta
(98% mortality) and Barranquilla (100% mortality), and possible development of resistance was detected in Valledupar (96.8% mortality) and Montería (93.2% mortality). The populations from Cartagena (87.9% mortality) and Chiriguana (86.0% mortality) were found to be resistant to deltamethrin (Fig 2).

Additionally, a total of 1822 adult female *Ae. aegypti* were tested in CDC bioassays for susceptibility to \( \lambda \)-cyhalothrin \( (n = 606) \), deltamethrin \( (n = 608) \), and permethrin \( (n = 608) \). Resistance to \( \lambda \)-cyhalothrin was detected in the populations from Barranquilla (79.6% mortality), Chiriguana (83.5% mortality), Juan de Acosta (71.6% mortality), and Montería (35% mortality), whereas the populations from Cartagena (98.0% mortality) and Valledupar (100% mortality) were susceptible (Fig 3A). Susceptibility to deltamethrin was observed in all populations, with mortalities of 100% (Fig 3B). Resistance to permethrin was detected in the populations from Juan de Acosta (80.0% mortality), Montería (69.0% mortality), and Barranquilla (64% mortality), and susceptibility was observed in the populations from Cartagena, Chiriguana and Valledupar, with mortalities of 100% (Fig 3C), (Fig 3).

In populations where resistance intensity was assessed, 100% mortality at the diagnostic time was observed after exposure to twice the concentration (2X) of the recommended diagnostic dose for \( \lambda \)-cyhalothrin (20 \( \mu \)g/bottle) and permethrin (30 \( \mu \)g/bottle) (Table 2).

### Biochemical assays

Based on the classification criteria of Montella *et al.* [40], \( \alpha \)-esterase enzyme levels were highly altered in the population from Montería, where 79% of individuals exceeded the 99th percentile of the Rockefeller reference population (Fig 4A). Similarly, \( \beta \)-esterase activity levels were highly altered in the population of Montería (97%) and were also altered in the populations from Juan de Acosta (45%), Barranquilla (31%), Valledupar (27%) and Cartagena (12%) (Fig 4B), and pNPA-esterase activity levels were altered in the population from Juan de Acosta.
(14%) (Fig 4C). Highly altered MFO activity levels were detected in the populations from Juan de Acosta (92%), Montería (97%), and Valledupar (88%) (Fig 4D). Altered GST activity levels were detected in the populations from Barranquilla (17%), Cartagena (24%), Juan de Acosta

Table 2. Mortality of *Ae. aegypti* exposed to 1X and 2X the diagnostic doses of λ-cyhalothrin and permethrin.

| Insecticide         | Populations    | 1× DD 10 µg/bottle | 2× DD 20 µg/bottle | 1× DD 15 µg/bottle | 2× DD 30 µg/bottle |
|---------------------|----------------|---------------------|--------------------|-------------------|-------------------|
| λ-cyhalothrin       |                |                     |                    |                   |                   |
| (DDa: 10 µg/bottle, DDb: 30 min) | Barranquilla   | 103                  | 76.61              | 100               | 100               |
|                     | Chiriquí       | 103                  | 83.49              | 100               | 100               |
|                     | Juan de Acosta| 102                  | 71.56              | 100               | 100               |
|                     | Montería      | 100                  | 35.0               | 100               | 100               |
| Permethrin          |                |                     |                    |                   |                   |
| (DDa: 15 µg/bottle, DDb: 30 min) | Barranquilla   | 100                  | 64.0               | 100               | 100               |
|                     | Juan de Acosta| 100                  | 80.0               | 100               | 100               |
|                     | Montería      | 100                  | 69.0               | 100               | 100               |

a Total number of females evaluated.
b Diagnostic dose.
c Diagnostic time.

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(44%), Montería (34%) and Chiriguana (4%) (Fig 4E). AChE activity remained unaltered in all populations evaluated (Fig 4F). Overall, significant differences were observed between the mean activity levels of most enzyme groups between the field populations and the Rockefeller reference strain ($p < 0.05$) (Fig 4).

**kdr allele frequencies**

All three kdr mutations were detected in all the populations evaluated. Regarding the V1016I mutation, all three genotypes ($V_{1016}V_{1016}$, $V_{1016}I_{1016}$ and $I_{1016}I_{1016}$) were detected in each field population. The mutant allele $I_{1016}$ was the most prevalent in the population from Montería, with a frequency of 0.70, and the least prevalent in the populations from Barranquilla and Valledupar, with a frequency of 0.15 in both. Regarding the F1534C mutation, all three genotypes ($F_{1534}F_{1534}$, $F_{1534}C_{1534}$ and $C_{1534}C_{1534}$) were detected in the populations from Barranquilla and Juan de Acosta, whereas only $F_{1534}$ and $C_{1534}$ were detected in Cartagena, Chiriguana, and Valledupar, with $C_{1534}$ predominating in all populations. It is noteworthy that the $C_{1534}$ genotype was fixed in the population from Montería with a frequency of 1.0 (Table 3). In addition, the frequency of the $C_{1534}$ mutant allele in the populations from Cartagena, Valledupar, and Chiriguana ranged between 0.94 and 0.97, but was 0.76 in the populations from Barranquilla and Juan de Acosta. Regarding the V410L mutation, all three genotypes ($V_{410}V_{410}$, $V_{410}L_{410}$, and $L_{410}L_{410}$) were detected in each field population. The highest frequency of the L410 allele was detected in Montería with a frequency of 0.72, whereas the lowest was detected in Valledupar with a
frequency of 0.05. For the other populations, the frequencies of the L410 allele ranged between 0.12 and 0.32 (Table 3).

For loci 1016 and 1534, all genotypes were found to be in Hardy–Weinberg equilibrium. In the case of locus 410, the genotypes of most populations, except Valledupar, were in Hardy–Weinberg equilibrium (p < 0.05). When determining the inbreeding coefficients (F<sub>IS</sub>) for I1016, values < 0 were obtained for the populations from Barranquilla and Valledupar due to an excess of heterozygotes, in contrast to the populations from Cartagena, Chiriguana, Juan de Acosta, and Montería, where values > 0 were recorded due to a deficiency of heterozygotes.

For C1534, a generalized excess of heterozygotes was observed, with the exception of Barranquilla, where a deficiency of heterozygotes was observed. Similarly, for L410, the populations from Barranquilla, Cartagena, and Valledupar showed a deficiency of heterozygotes, in contrast to Chiriguana, Juan de Acosta, and Montería, where an excess of heterozygotes was detected (Table 3).

Of the 27 combinations of tri-locus genotypes, 13 combinations were detected in 281 mosquitoes collected from the six evaluated populations. The triple homozygous wild-type genotype (VV<sub>1016</sub>/FF<sub>1534</sub>/VV<sub>410</sub>) was detected only in the populations from Barranquilla and Juan de Acosta, with frequencies of 0.08 and 0.04, respectively, whereas the triple homozygous mutant genotype (II<sub>1016</sub>/CC<sub>1534</sub>/LL<sub>410</sub>) was present in all populations except Valledupar, with frequencies between 0.02 (Barranquilla) and 0.49 (Montería). Similarly, the triple heterozygous

| Table 3. Genotype and allele frequencies of the V1016I, F1534C, and V410L kdr mutations in F0 Ae. aegypti females. |
|-------------------------------------------------------------|
| **Population** | n | Genotype frequency | Allele frequency | Hardy–Weinberg | F<sub>IS</sub> |
|----------------|---|-------------------|-----------------|----------------|----------|
|                |   | V1016I            |                 |                |          |
|                |   | VV | VI | II | V | I | χ² | p value |          |
| Barranquilla   | 49 | 0.71 | 0.27 | 0.02 | 0.85 | 0.15 | 0.02 | 0.87 | -0.02 |
| Cartagena      | 46 | 0.74 | 0.20 | 0.07 | 0.84 | 0.16 | 3.68 | 0.06 | 0.28 |
| Chiriguana     | 47 | 0.57 | 0.34 | 0.09 | 0.74 | 0.26 | 0.51 | 0.47 | 0.10 |
| Juan de Acosta | 48 | 0.75 | 0.23 | 0.04 | 0.86 | 0.16 | 0.88 | 0.34 | 0.15 |
| Montería       | 43 | 0.09 | 0.42 | 0.49 | 0.30 | 0.70 | 0.00 | 0.95 | 0.00 |
| Valledupar     | 48 | 0.73 | 0.25 | 0.02 | 0.85 | 0.15 | 0.00 | 0.98 | -0.00 |
|                |   | F1534C            |                 |                |          |
|                |   | N | FF | FC | CC | F | C | χ² | p value | F<sub>IS</sub> |
| Barranquilla   | 49 | 0.10 | 0.29 | 0.61 | 0.24 | 0.76 | 2.53 | 0.11 | 0.22 |
| Cartagena      | 46 | 0.00 | 0.07 | 0.93 | 0.03 | 0.97 | 0.05 | 0.82 | -0.03 |
| Chiriguana     | 47 | 0.00 | 0.11 | 0.89 | 0.05 | 0.95 | 0.15 | 0.70 | -0.05 |
| Juan de Acosta | 48 | 0.04 | 0.44 | 0.54 | 0.26 | 0.76 | 0.80 | 0.37 | -0.10 |
| Montería       | 43 | 0.00 | 0.00 | 1.00 | 0.00 | 1.00 | -   | -   | -   |
| Valledupar     | 48 | 0.00 | 0.13 | 0.87 | 0.06 | 0.94 | 0.21 | 0.64 | -0.07 |
|                |   | V410L             |                 |                |          |
|                |   | N | VV | VL | LL | V | L | χ² | p value | F<sub>IS</sub> |
| Barranquilla   | 49 | 0.02 | 0.20 | 0.78 | 0.88 | 0.12 | 0.12 | 0.72 | 0.05 |
| Cartagena      | 46 | 0.07 | 0.37 | 0.57 | 0.75 | 0.25 | 0.00 | 0.92 | 0.01 |
| Chiriguana     | 47 | 0.09 | 0.47 | 0.45 | 0.68 | 0.32 | 0.28 | 0.59 | -0.07 |
| Juan de Acosta | 48 | 0.02 | 0.27 | 0.71 | 0.84 | 0.16 | 0.03 | 0.85 | -0.02 |
| Montería       | 43 | 0.51 | 0.42 | 0.07 | 0.28 | 0.72 | 0.07 | 0.79 | -0.04 |
| Valledupar     | 48 | 0.04 | 0.02 | 0.94 | 0.95 | 0.05 | 29.88 | 0.00 | 0.79 |

* Number of mosquitoes evaluated.
F<sub>IS</sub>, inbreeding coefficient.

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genotype (VI\textsubscript{1016}/FC\textsubscript{1534}/VL\textsubscript{410}) was present only in Chiriguana and Juan de Acosta at low frequencies (0.02 and 0.06, respectively). The homozygous wild-type genotype for loci 1016 and 410 and homozygous resistant for locus 1534 (VV\textsubscript{1016}/CC\textsubscript{1534}/VV\textsubscript{410}) was most frequent in Barranquilla, Cartagena, Chiriguana, and Valledupar, with frequencies of 0.37, 0.54, 0.43, and 0.58, respectively; the exceptions were Juan de Acosta, where the most frequent genotype was homozygous wild-type for loci 1016 and 410 and heterozygous for locus 1534 (VV\textsubscript{1016}/FC\textsubscript{1534}/VV\textsubscript{410}) with a frequency of 0.33, and Montería, where the most frequent genotype was the triple homozygous mutant (II\textsubscript{1016}/CC\textsubscript{1534}/LL\textsubscript{410}), with a frequency of 0.49 (Fig 5).

**Association of kdr alleles with phenotypic resistance to pyrethroids**

Based on the results obtained with the mosquitoes exposed to insecticides in the WHO bioassays, a significant association (p < 0.05) was identified between the mutant kdr alleles 1016I, 1534C, and 410L and resistance to \(\lambda\)-cyhalothrin in the populations from Juan de Acosta, Montería, and Valledupar. Similarly, an association was observed between the 1534C allele and resistance to deltamethrin in the populations of Chiriguana, Montería, and Valledupar and between the 1016I and 410L alleles and resistance to deltamethrin in the population of Montería. A significant association (p < 0.05) was also detected between the 1016I, 1534C, and 410L alleles and resistance to permethrin in the populations from Chiriguana, Montería, and Valledupar; between the 1534C allele and resistance to permethrin in Barranquilla, Cartagena, and Juan de Acosta; and between the 410L allele and permethrin resistance in Juan de Acosta (Tables 4–6).
### Table 4. Association between 1016I, 1534C, and 410L alleles and resistance to λ-cyhalothrin in adult *Ae. aegypti* in WHO bioassays.

| kdr mutation | Genotype | n   | Phenotype | OR (95%CI) | p value |
|--------------|----------|-----|-----------|------------|---------|
|              |          | R² |           |            |         |
|              |          | S² |           |            |         |
| **Barranquilla** |          |    |           |            |         |
| V1016I       | VI       | 15 | 0.80      | 0.23–2.83  | 0.740   |
|              | VV       | 28 | 1.26      | 0.34–4.59  | 0.726   |
|              | CC       | 22 | 1.40      | 0.39–5.38  | 0.582   |
| F1534C       | FC       | 18 | 2.50      | 0.512–12.2 | 0.245   |
|              | FF       | 3  | 4.05      | 1.61–17.09 | 0.042   |
|              | LL       | 2  | 2.78      | 1.19–6.58  | 0.017   |
| V410L        | VL       | 12 | 0.74      | 0.18–2.99  | 0.670   |
|              | VV       | 31 | 3.02      | 1.28–7.11  | 0.009   |
|              | CC       | 35 | 6.46      | 2.38–17.51 | 0.000   |
|              | FF       | 1  |           |            |         |
|              | LL       | 4  |           |            |         |
| V410L        | VL       | 36 | 6.02      | 2.60–13.91 | 0.000   |
|              | VV       | 35 |           |            |         |
| **Cartagena** |          |    |           |            |         |
| V1016I       | VI       | 15 | 0.80      | 0.23–2.83  | 0.740   |
|              | VV       | 28 | 1.26      | 0.34–4.59  | 0.726   |
|              | CC       | 22 | 1.40      | 0.39–5.38  | 0.582   |
| F1534C       | FC       | 18 | 2.50      | 0.512–12.2 | 0.245   |
|              | FF       | 3  | 4.05      | 1.61–17.09 | 0.042   |
|              | LL       | 2  | 2.78      | 1.19–6.58  | 0.017   |
| V410L        | VL       | 12 | 0.74      | 0.18–2.99  | 0.670   |
|              | VV       | 31 | 3.02      | 1.28–7.11  | 0.009   |
|              | CC       | 35 | 6.46      | 2.38–17.51 | 0.000   |
|              | FF       | 1  |           |            |         |
|              | LL       | 4  |           |            |         |
| V410L        | VL       | 36 | 6.02      | 2.60–13.91 | 0.000   |
|              | VV       | 35 |           |            |         |
| **Chiriguaná** |          |    |           |            |         |
| V1016I       | VI       | 22 | 0.50      | 0.23–1.06  | 0.069   |
|              | VV       | 28 | 1.26      | 0.34–4.59  | 0.726   |
|              | CC       | 53 | 4.05      | 0.96–17.09 | 0.042   |
| F1534C       | FC       | 9  | 4.05      | 0.96–17.09 | 0.042   |
|              | FF       | 0  |           |            |         |
|              | LL       | 9  |           |            |         |
| V410L        | VL       | 21 | 0.97      | 0.44–2.15  | 0.948   |
|              | VV       | 32 | 3.02      | 1.28–7.11  | 0.009   |
|              | CC       | 35 | 6.46      | 2.38–17.51 | 0.000   |
|              | FF       | 1  |           |            |         |
|              | LL       | 4  |           |            |         |
| V410L        | VL       | 23 | 2.78      | 1.19–6.58  | 0.017   |
|              | VV       | 29 |           |            |         |
| **Juan de Acosta** |        |    |           |            |         |
| V1016I       | VI       | 26 | 3.02      | 1.28–7.11  | 0.009   |
|              | VV       | 27 | 1.40      | 0.39–5.38  | 0.582   |
|              | CC       | 35 | 5.14      | 1.61–16.40 | 0.003   |
| F1534C       | FC       | 20 | 5.14      | 1.61–16.40 | 0.003   |
|              | FF       | 1  |           |            |         |
|              | LL       | 4  |           |            |         |
| V410L        | VL       | 23 | 2.78      | 1.19–6.58  | 0.017   |
|              | VV       | 29 |           |            |         |
| **Montería** |          |    |           |            |         |
| V1016I       | VI       | 37 | 4.85      | 2.31–10.18 | 0.000   |
|              | VV       | 29 | 1.40      | 0.39–5.38  | 0.582   |
|              | CC       | 63 | 6.46      | 2.38–17.51 | 0.000   |
| F1534C       | FC       | 15 | 6.46      | 2.38–17.51 | 0.000   |
|              | FF       | 4  |           |            |         |
|              | LL       | 11 |           |            |         |
| V410L        | VL       | 36 | 6.02      | 2.60–13.91 | 0.000   |
|              | VV       | 35 |           |            |         |

(Continued)
Less association was detected between kdr alleles and the observed phenotype in the CDC bioassays. A significant association (p < 0.05) between the 1534C allele and resistance to λ-cyhalothrin was detected in the population from Barranquilla and between the 1016I and 410L alleles and resistance to permethrin in the population from Montería. Despite the resistance to λ-cyhalothrin detected in the population from Barranquilla, no significant association was found between resistance to deltamethrin and the 1534C allele in the population from Cartagena.

### Table 4. (Continued)

| kdr mutation | Genotype | n^a | Phenotype | OR^d (95%CI)^e | p value^f |
|--------------|----------|-----|-----------|---------------|-----------|
| Valledupar   | II       | 1   | R          | 1             |           |
|              | V1016I   | 5   | 13.62 (1.56–118.80) | *0.003 |
|              | VV       | 40  | 11         | 29            |           |
|              | CC       | 12  | 1          | 1             |           |
|              | F1534C   | 23  | 10.8 (3.61–32.28) | *0.000 |
|              | FF       | 11  | 0          | 11            |           |
|              | LL       | 1   | 1          | 0             |           |
|              | V410L    | 5   | 13.62 (1.56–118.8) | *0.003 |
|              | VV       | 40  | 11         | 29            |           |

^aSample size  
^bResistant mosquitoes  
^cSusceptible mosquitoes  
^dOdds ratio for the association between the mutant alleles 1016I, 1534C, and 410L and resistance to λ-cyhalothrin  
^eLower and upper limits of the confidence interval for the OR  
^fSignificant difference (p < 0.05)–Pearson X^2^.

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Less association was detected between kdr alleles and the observed phenotype in the CDC bioassays. A significant association (p < 0.05) between the 1534C allele and resistance to λ-cyhalothrin was detected in the population from Barranquilla and between the 1016I and 410L alleles and resistance to permethrin in the population from Montería. Despite the resistance to λ-cyhalothrin detected in the population from Barranquilla, no significant association was found between resistance to deltamethrin and the 1534C allele in the population from Cartagena.

### Table 5. Association between 1016I, 1534C, and 410L alleles and resistance to deltamethrin in adult Ae. aegypti in WHO bioassays.

| kdr mutation | Genotype | n^a | Phenotype | OR^d (95%CI)^e | p value^f |
|--------------|----------|-----|-----------|---------------|-----------|
| Cartagena    | II       | 1   | 0.52 (0.13–2.01) | 0.337 |
|              | V1016I   | 15  | 0.58 (0.20–1.66) | 0.312 |
|              | VV       | 25  | 17         |               |           |
|              | CC       | 22  | 18         |               |           |
|              | F1534C   | 15  | 6          | 9             |           |
|              | FF       | 1   | 3          |               |           |
|              | LL       | 1   | 0          |               |           |
|              | V410L    | 15  | 0.52 (0.13–2.01) | 0.337 |
|              | VV       | 25  | 17         |               |           |
| Chiriguana   | II       | 0   | 0          |               |           |
|              | V1016I   | 9   | 3.14 (0.74–13.26) | 0.105 |
|              | VV       | 30  | 25         |               |           |
|              | CC       | 11  | 5          |               |           |
|              | F1534C   | 21  | 3          | 18            |           |
|              | FF       | 7   | 0          | 7             |           |
|              | LL       | 0   | 0          |               |           |
|              | V410L    | 6   | 3          | 3             |           |
|              | VV       | 33  | 27         |               |           |

(Continued)
pyrethroids detected with the CDC bioassays in the populations from Chiriguana and Juan de Acosta, no significant associations were detected between \textit{kdr} alleles and resistant phenotypes in these populations (Tables 7 and 8).

### Comparisons of tri-locus genotypes with resistance to pyrethroids

Of the 27 possible combinations of genotypes, 20 combinations of tri-locus genotypes were detected in the 918 mosquitoes phenotyped in WHO bioassays. The most common haplotypes were VV\textsubscript{1016}/CC\textsubscript{1534}/VV\textsubscript{410} (n = 233 mosquitoes, 25.4%), VV\textsubscript{1016}/FC\textsubscript{1534}/VV\textsubscript{410} (n = 198, 21.6%), and VI\textsubscript{1016}/CC\textsubscript{1534}/VL\textsubscript{410} (n = 187, 20.4%). Wild-type double homozygotes at loci 1016 and 410 in the presence of CC\textsubscript{1534}/FC\textsubscript{1534} were significantly more likely to be phenotypically

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**Table 5.** (Continued)

| \(kdr\) mutation | Genotype | \(n^a\) | Phenotype | \(\text{OR}^d\) (95%CI)\(^e\) | \(p\) value\(^f\) |
|------------------|----------|--------|-----------|-----------------|-----------------|
| **Juan de Acosta** |          |        |           |                 |                 |
|                  | II       | 0      | 1         | 0               |                 |
| V1016I           | VI       | 16     | 1         | 15              | 9 (0.90–93.17)  | 0.031           |
|                  | VV       | 15     | 0         | 15              |                 |
|                  | CC       | 20     | 2         | 18              |                 |
| F1534C           | FC       | 9      | 0         | 9               | 0.0             | 0.253           |
|                  | FF       | 3      | 0         | 3               |                 |
|                  | LL       | 1      | 1         | 0               |                 |
| V410L            | VL       | 14     | 0         | 14              | 3.29 (0.42–25.50) | 0.233           |
|                  | VV       | 17     | 1         | 16              |                 |
| **Montería**     |          |        |           |                 |                 |
|                  | II       | 5      | 4         | 1               |                 |
| V1016I           | VI       | 16     | 4         | 12              | 6.57 (2.08–20.71) | *0.000          |
|                  | VV       | 18     | 1         | 17              |                 |
|                  | CC       | 29     | 9         | 20              |                 |
| F1534C           | FC       | 8      | 0         | 8               | 0.0             | *0.039          |
|                  | FF       | 2      | 0         | 2               |                 |
|                  | LL       | 5      | 4         | 1               |                 |
| V410L            | VL       | 18     | 4         | 14              | 5.5 (1.77–17.11) | *0.001          |
|                  | VV       | 16     | 1         | 15              |                 |
| **Valledupar**   |          |        |           |                 |                 |
|                  | II       | 0      | 0         | 0               |                 |
| V1016I           | VI       | 3      | 0         | 3               | 0.0             | 0.517           |
|                  | VV       | 31     | 4         | 27              |                 |
|                  | CC       | 5      | 3         | 2               |                 |
| F1534C           | FC       | 16     | 0         | 16              | 6 (1.11–32.45)  | *0.022          |
|                  | FF       | 13     | 1         | 12              |                 |
|                  | LL       | 0      | 0         | 0               |                 |
| V410L            | VL       | 3      | 0         | 3               | 0.0             | 0.517           |
|                  | VV       | 31     | 4         | 27              |                 |

\(^a\)Sample size

\(^b\)Resistant mosquitoes

\(^c\)Susceptible mosquitoes

\(^d\)Odds ratio for the association between the mutant alleles 1016I, 1534C, and 410L and resistance to deltamethrin

\(^e\)Lower and upper limits of the confidence interval for the OR

\(^f\)Significant difference (\(p < 0.05\))–Pearson \(X^2\).
Table 6. Association between 1016I, 1534C, and 410L alleles and resistance to permethrin in adult *Ae. aegypti* in WHO bioassays.

| *kdr* mutation | Genotype | n<sup>a</sup> | Phenotype | OR<sup>b</sup> (95%CI)<sup>c</sup> | p value<sup>d</sup> |
|---------------|----------|----------------|------------|-------------------------------|-----------------|
|               |          |                |            |                              |                 |
| **Barranquilla** |          |                |            |                              |                 |
| V1016I        | VI       | 18             | 11         | 7                             | 1.78 (0.66–4.78) | 0.249           |
|               | VV       | 30             | 17         | 13                            |                 |
|               | CC       | 35             | 22         | 13                            |                 |
|               | F1534C   | FC             | 20         | 8                             | 3.01 (1.28–7.09) | 0.009           |
|               |          | FF             | 6          | 1                             | 3.01 (1.28–7.09) | 0.009           |
|               |          | LL             | 1          | 1                             |                 |
| V410L         | VL       | 19             | 12         | 7                             | 2.21 (0.82–5.93) | 0.110           |
|               | VV       | 41             | 18         | 23                            |                 |
| **Cartagena** |          |                |            |                              |                 |
| V1016I        | VI       | 19             | 10         | 9                             | 0.57 (0.62–3.98) | 0.337           |
|               | VV       | 32             | 12         | 20                            |                 |
|               | CC       | 32             | 17         | 15                            |                 |
|               | F1534C   | FC             | 17         | 6                             | 3.09 (1.12–8.53) | 0.025           |
|               |          | FF             | 4          | 0                             |                 |
|               |          | LL             | 2          | 1                             |                 |
| V410L         | VL       | 18             | 9          | 9                             | 1.4 (0.55–3.59)  | 0.482           |
|               | VV       | 33             | 13         | 20                            |                 |
| **Chiriguaná** |          |                |            |                              |                 |
| V1016I        | VI       | 20             | 18         | 2                             | 13.92 (3.13–61.84) | 0.000           |
|               | VV       | 44             | 16         | 28                            |                 |
|               | CC       | 21             | 18         | 3                             |                 |
|               | F1534C   | FC             | 33         | 18                            | 5.01 (2.40–10.50) | 0.000           |
|               |          | FF             | 13         | 1                             |                 |
|               |          | LL             | 4          | 4                             |                 |
| V410L         | VL       | 17             | 16         | 1                             | 28.32 (3.70–216.80) | 0.000           |
|               | VV       | 46             | 17         | 29                            |                 |
| **Juan de Acosta** |      |                |            |                              |                 |
| V1016I        | VI       | 26             | 17         | 9                             | 1.58 (0.78–3.20) | 0.201           |
|               | VV       | 30             | 14         | 16                            |                 |
|               | CC       | 48             | 32         | 16                            |                 |
|               | F1534C   | FC             | 20         | 7                             | 3.38 (1.27–8.93) | 0.010           |
|               |          | FF             | 1          | 0                             |                 |
|               |          | LL             | 7          | 6                             |                 |
| V410L         | VL       | 30             | 19         | 11                            | 2.38 (1.11–5.12) | 0.023           |
|               | VV       | 32             | 14         | 18                            |                 |
| **Montería**  |          |                |            |                              |                 |
| V1016I        | VI       | 29             | 24         | 5                             | 2.99 (1.40–6.35) | 0.003           |
|               | VV       | 40             | 24         | 16                            |                 |
|               | CC       | 70             | 64         | 6                             |                 |
|               | F1534C   | FC             | 16         | 4                             | 33 (10.51–103.60) | 0.000           |
|               |          | FF             | 6          | 0                             |                 |
|               |          | LL             | 21         | 20                            |                 |
| V410L         | VL       | 25             | 21         | 4                             | 5.69 (2.27–14.28) | 0.000           |
|               | VV       | 46             | 27         | 19                            |                 |

(Continued)
susceptible to deltamethrin ($p < 0.05$). Heterozygotes at both loci 1016 and 410 in the presence of CC1534 were significantly more likely to be resistant to $\lambda$-cyhalothrin and permethrin ($p < 0.05$) and susceptible to deltamethrin ($p < 0.05$) (Table 9).

From the CDC bioassays, 15 combinations of tri-locus genotypes were observed in 465 mosquitoes assayed with $\lambda$-cyhalothrin and permethrin in Barranquilla, Juan de Acosta, and Montería. Similar to the WHO bioassays, the most common haplotypes were VI1016/CC1534/VL410 ($n = 161, 34.6\%$) and VV1016/CC1534/VV410 ($n = 117, 25.2\%$). Wild-type double homozygotes at loci 1016 and 410 in the presence of CC1534/FC1534 were significantly more likely to be phenotypically susceptible to $\lambda$-cyhalothrin and permethrin ($p < 0.05$) (Table 10).

### Discussion

In Colombia, the use of pyrethroids for the control of Ae. aegypti is a fairly recent phenomenon. Among the pyrethroids, $\lambda$-cyhalothrin and deltamethrin have most commonly been used to control Ae. aegypti in Colombia. However, resistance to $\lambda$-cyhalothrin has been more commonly reported than resistance to deltamethrin in Colombia, as demonstrated by results from previous studies [7, 9–11, 13, 26] as well as those obtained in the present study. In the findings presented here, we detected resistance to permethrin and $\lambda$-cyhalothrin in all populations and varying degrees of susceptibility to deltamethrin. This heterogeneity of resistance patterns within the pyrethroid class suggests that diverse mechanisms are contributing to these phenotypes.

Resistance to DDT is widespread in Colombia owing to the application of this organochlorine compound for more than five decades in the country [20]. DDT and pyrethroids share the mode of action consisting of delayed sodium channel closure and membrane repolarization [41]. The modification of this target site due to the presence of $kdr$ mutations on the para gene can lead to cross-resistance to both DDT and pyrethroids. As such, the high prevalence of $kdr$ alleles detected in our study may also be linked to previous selection pressures caused by DDT [42, 43].
Table 7. Association between 1016I, 1534C, and 410L alleles and resistance to λ-cyhalothrin in adult *Ae. aegypti* in CDC bioassays.

|                |          | Genotype | n^ | Phenotype | OR (95%CI)^^ | p value^7 |
|----------------|----------|----------|----|-----------|--------------|-----------|
|                | kdr mutation |          |    |           |              |           |
| Barranquilla   | II       |          | 0  | 0         |              |           |
| V1016I         | VI       |          | 16 | 9         | 2.06 (0.70–0.18) | 0.182    |
|                | VV       |          | 35 | 12        |              |           |
|                | CC       |          | 27 | 16        |              |           |
|                | F1534C   | FC       | 21 | 5         | 4.28 (1.47–12.51) | *0.005   |
|                |          | FF       | 3  | 0         |              |           |
|                |          | LL       | 0  | 0         |              |           |
| V410L          | VL       |          | 16 | 9         | 2.06 (0.70–6.07) | 0.182    |
|                | VV       |          | 35 | 12        |              |           |
| Chiriguana     | II       |          | 6  | 3         |              |           |
| V1016I         | VI       |          | 22 | 7         | 1.15 (0.48–2.75) | 0.753    |
|                | VV       |          | 19 | 7         |              |           |
|                | CC       |          | 43 | 16        |              |           |
|                | F1534C   | FC       | 4  | 1         | 1.73 (0.17–17.38) | 0.634    |
|                |          | FF       | 0  | 0         |              |           |
|                |          | LL       | 6  | 3         |              |           |
| V410L          | VL       |          | 24 | 9         | 1.47 (0.62–3.46) | 0.382    |
|                | VV       |          | 17 | 5         |              |           |
| Juan de Acosta | II       |          | 4  | 3         |              |           |
| V1016I         | VI       |          | 21 | 11        | 1.94 (0.82–4.58) | 0.126    |
|                | VV       |          | 31 | 12        |              |           |
|                | CC       |          | 29 | 14        |              |           |
|                | F1534C   | FC       | 23 | 10        | 1.07 (0.47–2.46) | 0.867    |
|                |          | FF       | 4  | 2         |              |           |
|                |          | LL       | 4  | 2         |              |           |
| V410L          | VL       |          | 24 | 12        | 1.22 (0.54–2.78) | 0.631    |
|                | VV       |          | 28 | 12        |              |           |
| Montería       | II       |          | 14 | 10        |              |           |
| V1016I         | VI       |          | 51 | 35        | 1.41 (0.77–2.57) | 0.269    |
|                | VV       |          | 35 | 20        |              |           |
|                | CC       |          | 98 | 63        |              |           |
|                | F1534C   | FC       | 1  | 1         | 0.30 (0.01–6.12) | 0.409    |
|                |          | FF       | 1  | 1         |              |           |
|                |          | LL       | 15 | 10        |              |           |
| V410L          | VL       |          | 50 | 34        | 1.20 (0.66–2.18) | 0.545    |
|                | VV       |          | 35 | 21        |              |           |

^Sample size

^Resistant mosquitoes

^Susceptible mosquitoes

^Odds ratio for the association between the mutant alleles 1016I, 1534C, and 410L and resistance to λ-cyhalothrin

^Lower and upper limits of the confidence interval for the OR

^Significant difference (p < 0.05).

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Our findings of resistance to \( \lambda \)-cyhalothrin in populations of *Ae. aegypti* in the Caribbean region of Colombia are consistent with those reported by Maestre *et al.* [13] for the populations of Barranquilla and Montería, Granada *et al.* [15] for the population of Riohacha, and Atencia *et al.* [31] for the population of Sincelejo. Likewise, Maestre *et al.* [13] reported resistance to \( \lambda \)-cyhalothrin in Valledupar and moderate resistance in Cartagena, while in the present work we detected resistance using the WHO bioassay but susceptibility using the CDC bioassay in both populations. However, other studies in Colombia have found resistance to \( \lambda \)-cyhalothrin using both the WHO and CDC techniques in the departments of Cundinamarca, Caquetá, Meta, Guaviare, Santander, Chocó, Antioquia, Putumayo and Casanare [9, 10, 12]. Ocampo *et al.* [11] found susceptibility to this insecticide using the CDC technique in the departments of Cauca, Nariño, Valle del Cauca and Huila.

| Permethrin | Genotype | n | Phenotype | OR\(^d\) (95%CI)\(^e\) | p value\(^f\) |
|------------|----------|---|-----------|---------------------|-----------|
| Barranquilla | II | 4 | 3 | 1 |  |
| V1016I | VI | 36 | 14 | 22 | 1.67 (0.84–3.29) | 0.139 |
| | VV | 60 | 29 | 41 |  |
| | CC | 54 | 21 | 33 |  |
| F1534C | FC | 35 | 10 | 25 | 1.05 (0.55–2.01) | 0.865 |
| | FF | 11 | 5 | 6 |  |
| | LL | 3 | 2 | 1 |  |
| V410L | VL | 33 | 14 | 19 | 1.70 (0.83–3.45) | 0.141 |
| | VV | 64 | 20 | 44 |  |
| Juan de Acosta | II | 2 | 1 | 1 |  |
| V1016I | VI | 21 | 9 | 12 | 1.25 (0.50–3.12) | 0.637 |
| | VV | 27 | 10 | 17 |  |
| | CC | 24 | 9 | 15 |  |
| F1534C | FC | 24 | 9 | 15 | 0.69 (0.29–1.67) | 0.413 |
| | FF | 2 | 2 | 0 |  |
| | LL | 2 | 1 | 1 |  |
| V410L | VL | 21 | 9 | 12 | 1.25 (0.50–3.12) | 0.637 |
| | VV | 27 | 10 | 17 |  |
| Montería | II | 11 | 7 | 4 |  |
| V1016I | VI | 36 | 21 | 15 | 2.08 (1.01–4.30) | *0.045 |
| | VV | 14 | 3 | 11 |  |
| | CC | 61 | 31 | 30 |  |
| F1534C | FC | 0 | 0 | 0 | 1.03 (0.02–52.91) | 0.987 |
| | FF | 0 | 0 | 0 |  |
| | LL | 11 | 7 | 4 |  |
| V410L | VL | 36 | 21 | 15 | 2.08 (1.01–4.30) | *0.045 |
| | VV | 14 | 3 | 11 |  |

\(^a\)Sample size  
\(^b\)Resistant mosquitoes  
\(^c\)Susceptible mosquitoes  
\(^d\)Odds ratio for the association between the mutant alleles 1016I, 1534C, and 410L and resistance to \( \lambda \)-cyhalothrin  
\(^e\)Lower and upper limits of the confidence interval for the OR  
\(^f\)Significant difference (p < 0.05).  

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Table 9. Tri-locus genotypes of phenotyped adult *Ae. aegypti* from the six study populations after WHO bioassay.

| Insecticide | Phenotype | n<sup>c</sup> | II/CC/LL | II/FC/LL | VI/CC/LL | II/CC/VL | VI/FC/LL | VV/CC/LL | II/CC/VL | VI/FC/VL | VV/CC/VV | II/FC/VV | VI/VL | VV/FL | II/VL | VV/VC | II/FF/VL | VV/FF/VL | VV/FF/VV |
|-------------|-----------|--------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|-------|-------|-------|-------|----------|----------|----------|
| λ cyhalothrin | R<sup>a</sup> | 158 | 22 | 0 | 0 | 1 | 0 | 0 | 0 | 56 | 0 | 7 | 0 | 0 | 7 | 0 | 0 | 0 | 0 | 47 | 0 | 1 | 14 | 3 |
| | S<sup>b</sup> | 172 | 4 | 0 | 0 | 5 | 1 | 0 | 0 | 18 | 2 | 6 | 0 | 0 | 18 | 0 | 0 | 0 | 0 | 0 | 0 | 47 | 0 | 0 | 53 | 18 |
| Deltamethrin | R | 35 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 8 | 0 | 1 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 1 | 10 | 0 | 0 | 6 | 2 |
| | S | 150 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 14 | 0 | 0 | 1 | 0 | 3 | 31 | 1 | 0 | 42 | 26 |
| Permethrin | R | 229 | 33 | 0 | 1 | 0 | 4 | 1 | 0 | 66 | 0 | 8 | 3 | 0 | 19 | 0 | 0 | 1 | 0 | 2 | 60 | 0 | 0 | 29 | 2 |
| | S | 174 | 2 | 0 | 1 | 3 | 0 | 0 | 1 | 9 | 0 | 2 | 0 | 1 | 21 | 0 | 1 | 0 | 1 | 2 | 38 | 0 | 0 | 54 | 38 |
| Total | 918 | 68 | 0 | 2 | 13 | 2 | 0 | 1 | 187 | 2 | 24 | 3 | 1 | 81 | 0 | 1 | 2 | 1 | 8 | 233 | 1 | 1 | 198 | 89 |

<sup>a</sup>Resistant (living)

<sup>b</sup>Susceptible (dead)

<sup>c</sup>Total number of mosquitoes. The order of the genotypes is shown for loci 1016/1534/410. Resistant allele at locus 1016 = I, 1534 = C, 410 = L, triple-resistant genotype II/CC/LL, triple-susceptible genotype VV/FF/VV. Significant differences between resistant and susceptible are shown in bold (p < 0.05).

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Table 10. Tri-locus genotypes of phenotyped adult *Ae. aegypti* after CDC bioassay.

| Insecticide | Phenotype | n  | II/CC/LL | II/FC/LL | VI/CC/LL | VI/CC/VL | VI/FC/LL | VI/FC/VL | VI/CC/LL | VI/CC/VV | VI/FC/LL | VI/FC/VV | VI/VV/LL | VI/VV/VL | VI/VV/VV | VI/VV/FF | VI/VV/VF | VI/VV/VV |
|-------------|-----------|----|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| λ cyhalothrin | R<sup>a</sup> | 129 | 14 | 0 | 1 | 2 | 0 | 0 | 0 | 52 | 0 | 2 | 3 | 0 | 5 | 0 | 1 | 1 | 0 | 1 | 35 | 0 | 0 | 10 | 2 |
|             | S<sup>b</sup> | 125 | 7 | 0 | 1 | 1 | 0 | 2 | 0 | 41 | 0 | 1 | 1 | 0 | 5 | 0 | 0 | 2 | 0 | 0 | 33 | 0 | 0 | 25 | 6 |
| Permethrin   | R         | 87  | 10  | 0  | 0  | 1  | 0  | 0  | 0  | 34  | 0  | 1  | 0  | 0  | 9  | 0  | 0  | 0  | 0  | 0  | 15 | 0 | 0 | 10 | 7 |
|             | S         | 124 | 6   | 0  | 0  | 0  | 0  | 0  | 0  | 34  | 0  | 3  | 1  | 0  | 9  | 0  | 0  | 1  | 0  | 3  | 34 | 1 | 0 | 27 | 5 |
| Total       |           | 465 | 37  | 2  | 4  | 0  | 2  | 0  | 161 | 0  | 7  | 5  | 0  | 28 | 0  | 1  | 4  | 0  | 4  | 117 | 1 | 0 | 72 | 20 |

<sup>a</sup>Resistant (alive)

<sup>b</sup>Susceptible (dead)

<sup>c</sup>Total number of mosquitoes. The order of the genotypes is shown for loci 1016/1534/410. Resistant allele at locus 1016 = I, 1534 = C, 410 = L, triple-resistant genotype II/CC/LL, triple-susceptible genotype VV/FF/VV.

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Our findings of resistance to permethrin were consistent with those reported by Maestre et al. [13] for Barranquilla and Montería; however, for Cartagena and Valledupar, Maestre et al. had previously reported susceptibility, while we observed resistance using the WHO bioassay but susceptibility using the CDC bioassay. For this same insecticide Ardila et al. [12] encountered resistance in the department of Casanare, as did Fonseca et al. [10] in the departments of Chocó Antioquia and Putumayo using both bioassay methodologies. The results of the intensity bioassays for both permethrin and λ-cyhalothrin for the populations that had shown resistance using the CDC methodology resulted in 100% mortality when the diagnostic dose was doubled, suggesting that while resistance was present in the populations, it had not yet reached a high level of intensity.

For deltamethrin, Maestre et al. [13] had reported resistance in Barranquilla; however, we found susceptibility using both bioassay methodologies. In Montería and Valledupar, Maestre et al. [13] reported resistance to deltamethrin in both populations, while we found susceptibility with the CDC bioassay and indications that resistance was developing with the WHO bioassay. In Cartagena, Maestre et al. [13] had reported moderate resistance to deltamethrin, while we observed resistance with the WHO bioassay and susceptibility with the CDC bioassay.

Other studies carried out in Colombia showed resistance in *Ae. aegypti* to deltamethrin in the departments of Cundinamarca and Santander and susceptibility in the departments of Caquetá and Meta [9], as well as susceptibility in the departments of Cauca, Nariño, Valle del Cauca, Huila [11], Casanare [12] and Caldas [14] using the two bioassay methodologies.

In Colombia, both the CDC bioassay methodology and the WHO bioassay methodology have been used for insecticide susceptibility studies in adult *Ae. aegypti*. Typically, using both techniques, resistance to DDT has been observed in all *Ae. aegypti* populations evaluated in the country, together with variable susceptibility to pyrethroids and susceptibility to organophosphates in most populations [7]. In the present study, some discrepancies were observed between the results obtained with the WHO and CDC bioassay methodologies, indicating that the two techniques may not always provide consistent results. In studies by Aizoun et al. [44] and Fonseca et al. [20], WHO and CDC bioassays were compared to determine the susceptibility of *Anopheles gambiae* to deltamethrin and *Anopheles nuíeztovari* to fenitrothion. Both studies reported susceptibility when using the WHO bioassay and resistance when using the CDC bioassay. The authors observed that the exposure time of the mosquitoes to the insecticide (diagnostic time) was considerably shorter in the case of the CDC bioassay, which could have led to an overestimation of resistance; although in fact the opposite was observed in our study. Despite the shorter exposure time in the CDC bioassay, populations that were classified as resistant in the WHO bioassay were classified as susceptible in the CDC bioassay. This could potentially be explained due to the mechanisms underlying the resistance; for example, resistance that is primarily caused by *kdr* would likely result in populations that are not quickly knocked down and thus scored as ‘resistant’ at 30 minutes. However, if the main mechanisms of resistance are metabolic, mosquitoes may initially be knocked down but could recover over time as their detoxification enzymes metabolize the insecticide. Indeed, our biochemical assay data suggest that elevated enzymatic activity is present in the populations that were studied.

Most previous studies regarding enzymatic activity have been conducted on *Ae. aegypti* populations from other regions of Colombia where alterations were detected, mainly in MFOs and nonspecific esterases, in populations from Antioquia, Chocó, Putumayo, Cauca, Valle del Cauca, Nariño, Huila, Santander, Meta, and Casanare [9–12]. The one previous study conducted in the Caribbean region of Colombia reported altered α-esterases and MFOs in *Ae. aegypti* from Valledupar, MFOs in Cienaga, and GSTs in Sincelejo. In that previous study, no alterations in enzyme activity were detected in Cartagena, Montería, Barranquilla, San Juan, Puerto Colombia, and Soledad, [13]. Our results are consistent with the finding of highly
altered MFOs in Valledupar, and we also detected altered β-esterases in that same population. We also detected highly altered α-esterases, β-esterases, MFOs and GSTs in Montería; altered β-esterases and GSTs in Barranquilla; and altered GSTs in Cartagena. Additionally, in the present study we detected altered pNPA-esterases in the population of Juan de Acosta.

Regarding esterases, studies to date have reported the overexpression of β-esterases in populations resistant to organophosphates and pyrethroids [9–11]. Altered levels of α-esterase activity were detected previously in Valledupar in the study conducted by Maestre et al. [13]. In other countries, altered α-esterases, β-esterases, and MFOs have been reported in *Ae. aegypti* populations resistant to organophosphates, carbamates, and pyrethroids [40, 45–51].

There are no studies in Colombia incriminating insensitive acetylcholinesterase as a mechanism associated with resistance to organophosphates and carbamates in *Ae. aegypti*. A study by Grisales et al. [24] reported resistance to temephos in the population of *Ae. aegypti* from Cúcuta (RR: 15X) without evidence of insensitive acetylcholinesterase, although they did detect esterase and oxidase-based mechanisms.

*Kdr* mutations are important mechanisms involved in DDT and pyrethroid resistance. In Colombia, the first *kdr* mutation reported in populations of *Ae. aegypti* was V1016I, which was identified in populations from Puerto Colombia, Soledad, Barranquilla, Valledupar, San Juan, Sincelejo, Montería, Cienaga and Cartagena, which are all located in the Caribbean region. In that initial report, the V1016I mutation showed frequencies ranging between 0.07 and 0.35; the lowest frequency was found in the Cienaga population and the highest was found in Soledad, Montería, and Barranquilla, with frequencies of 0.35, 0.33, and 0.32, respectively [13]. The highest frequency of 1016I that we detected in the present study was in Montería, with a frequency of 0.70, showing a large increase in the frequency in this population from what was originally reported by Maestre et al. [13]. In addition, an increase in the frequency of 1016I from 0.09 to 0.16 was detected in Cartagena and a reduced frequency was detected in Barranquilla and Valledupar, from 0.32 and 0.27, respectively, to 0.15 in both populations. V1016I had also previously been reported in Quindío at low levels of frequency (0.02–0.05) [25].

The F1534C mutation was first detected in Colombia in the department of Sincelejo (Sucre), in the Caribbean region [31]. It had also previously been reported in *Ae. aegypti* populations from Puerto Colombia, Soledad, Barranquilla, Valledupar, San Juan, Sincelejo, Montería, Cienaga and Cartagena with frequencies ranging between 0.74 and 0.88. When compared with the results reported previously, we observed increased frequencies of 1534C, having risen in Barranquilla from 0.74 to 0.76, in Cartagena from 0.86 to 0.97, in Montería from 0.88 to 1.00, and in Valledupar from 0.82 to 0.94. These increases are likely attributable to the constant pressure exerted by pyrethroid insecticides, which were heavily applied during the period between the two studies for the control of dengue, chikungunya, and Zika. Although there are no previous studies reporting this mutation in Juan de Acosta and Chiriguana, these populations also showed high frequencies (0.76 and 0.95, respectively). Moreover, high frequencies of 1534C have been reported in other areas of Colombia, including Villavicencio, Riohacha, and Bello, with frequencies of 0.63, 0.71, and 0.56, respectively [15]. In these latter three populations, the V410L mutation was also identified in Colombia for the first time, with frequencies of 0.46, 0.30, and 0.06, respectively. It is noteworthy that in that study, *Ae. aegypti* from Bello were susceptible to λ-cyhalothrin, whereas those from Riohacha and Villavicencio were resistant. In these latter two populations, the researchers detected a positive association between V410L and V1016I and resistance to λ-cyhalothrin. In the present study, the V410L mutation was detected for the first time in the study populations, with frequencies ranging between 0.05 in Valledupar and 0.72 in Montería. The frequencies of the V1016I mutation were very similar to those of the V410L mutation in all the evaluated populations; this result is consistent with the findings reported by Granada et al. [15] for *Ae. aegypti* in Bello, Villavicencio, and Riohacha.
Haddi et al. [39] reported the presence of the V410L mutation in resistant *Ae. aegypti* in Brazil and observed that this mutation, either alone or in combination with the F1534C mutation, was strongly associated with increased resistance to type I and II pyrethroids. This is consistent with the results of the present study, where the 1534C and 410L alleles were associated with resistance to permethrin in the population of Juan de Acosta. The 1016I, 1534C, and 410L alleles were all associated with resistance to permethrin in the Chiriguaná, Montería, and Valledupar populations based on phenotyping by the WHO bioassay. In addition, F1534C was associated with resistance to deltamethrin in Chiriguaná, Valledupar, and Montería; V1016I and V410L were also associated with deltamethrin resistance in the case of the latter population. Similarly, an association was found between all three mutations and resistance to \( \lambda \)-cyhalothrin in Valledupar, Montería, and Juan de Acosta. This last result is consistent with the results of the study by Maestre et al. [32] which detected a significant positive correlation between the frequency of the 1016I allele and resistance to permethrin, \( \lambda \)-cyhalothrin, and cyfluthrin. However, no significant correlation was observed in that same study between 1534C and resistance to any pyrethroids [32].

Recent studies conducted in Mexico proposed three sequential models to explain the evolution of the V1016I, F1534C, and V410L mutations. The first model suggests that F1534C appeared first, providing low resistance levels, followed by the appearance of V1016I, which provided higher levels of resistance. The second model challenges the first model and proposes that V410L and V1016I occurred independently on a C1534 haplotype followed by cis conversion by recombination. Finally, a third model assumes that the three mutations appeared independently at low frequencies and that two recombination events rearranged them in a cis configuration [52]. Considering these previous models and the results obtained in the present investigation, it is possible to hypothesize that the appearance of V410L and V1016I did not occur independently because their allelic frequencies were so similar and they almost always appeared together.

Regarding the 1016/1534/410 phenotype–genotype association, a relationship between the VI\(_{1016}/CC_{1534}/VL_{410}\) genotype and resistance to \( \lambda \)-cyhalothrin and permethrin was detected in the present study. These results are consistent with the study conducted by Haddi et al. [39] in a pyrethroid-resistant *Ae. aegypti* strain from Brazil, where V410L alone or in combination with F1534C was shown to reduce sodium channel sensitivity to type I (permethrin) and type II pyrethroids (\( \lambda \)-cyhalothrin and deltamethrin). In addition, these results further support the notion that the presence of VI\(_{1016}\) and VL\(_{410}\) heterozygotes is sufficient to confer resistance to deltamethrin [52]. These findings suggest that the interactions of multiple mutations play a role in the response of *Ae. aegypti* sodium channels to insecticides [53].

In Colombia, previous studies have identified the frequency of V1016I, F1534C and V410L in populations of *Ae. aegypti* and have correlated those frequencies with the results of outcomes obtained through the CDC bioassay technique for populations from Puerto Colombia, Soledad and Barranquilla (Atlántico); Valledupar and San Juan (Cesar); Sincelejo (Sucre); Montería (Córdoba); Cienaga (Magdalena); Cartagena (Bolivar) [32]; Villavicencio (Meta); Riohacha (La Guajira); Bello (Antioquia) [15]; Giron (Santander); Buga, Palmira, Yumbo, Cali (Valle del Cauca) and Medellin (Antioquia) [54]. Unlike the aforementioned studies, our study evaluated for the first time for Colombia the direct association between phenotype and genotype in individual mosquitoes phenotyped through CDC and WHO bioassays. Furthermore, the present study established for the first time for Colombia differences between the phenotypes observed in CDC and WHO bioassays for pyrethroids and trilocus *kdr* haplotypes present in populations of *Ae. aegypti* from Montería (Cordoba); Cartagena (Bolivar); Juan de Acosta, Barranquilla (Atlántico); Chiriguaná and Valledupar (Cesar). This contributes to knowledge about the role and co-occurrence of these mutations in *Ae. aegypti* from the
Caribbean region and how they relate to phenotypic resistance, providing further evidence to
guide the selection of insecticides to be used in *Ae. aegypti* control.

A key strength of the present study is that it expands the knowledge base regarding the sus-
ceptibility status of *Ae. aegypti* to pyrethroid insecticides in Colombia. Additionally, it provides
new information regarding the frequency and distribution of *kdr* mutations and a detailed
analysis of phenotype-genotype associations. An increased understanding of the role of the
mechanisms involved in resistance will contribute to improved resistance surveillance strate-
gies which can better guide control programs for the selection of insecticides for the control of
*Ae. aegypti*. An important limitation of the current study is the lack of synergist bioassays,
which would permit an estimation of the relative contribution of metabolic mechanisms as
compared to *kdr* in conferring phenotypic resistance.

Regardless, the results presented here provide important input for territorial and national
entities in vector control decision-making. In addition, the detailed information on resistance
mechanisms provide deeper insight into the types of resistance mitigation and management
strategies might be most effective in the populations evaluated.

**Conclusions**

Variability was observed in pyrethroid susceptibility using the WHO and CDC bioassay meth-
 hodologies, highlighting the importance of using a consistent methodology to routinely screen
populations for susceptibility. The altered activity levels of β-esterases, α-esterases, MFOs, and
GSTs suggest that metabolic resistance may be important in these populations. The *kdr* muta-
tions V1016I, F1534C, and V410L were detected in all populations, with 1534C being nearly
fixed in all except two populations. Finally, associations were observed between the F1534C
mutation and resistance to permethrin in all populations, the F1534C mutation with resistance
to deltamethrin in Chiriguana, Montería, and Valledupar, and the V1016I, F1534C, and V410L
mutations and resistance to λ-cyhalothrin in Juan de Acosta, Valledupar, and Montería.

**Supporting information**

S1 Table. Mortality of populations of *Ae. aegypti* evaluated against diagnostic concentra-
tions of pyrethroid insecticides following WHO methodology.
(XLSX)

S2 Table. Mortality of populations of *Ae. aegypti* evaluated against diagnostic doses of
pyrethroid insecticides following CDC methodology.
(XLSX)

S3 Table. Enzymatic activity levels *Aedes aegypti* populations the study and Rockefeller
strain. a: insensitive acetylcholinesterase, b: Total protein, c: glutathione-S-transferases, d: α-
esterases, e: β-esterases, f: pNPA- esterases, g: mixed-function oxidases.
(XLSX)

S4 Table. Summary of frequencies of the 13 tri-locus genotypes present in F0 *Ae. aegypti*
 females. The order of the genotypes is 1016/1534/410. Mutant alleles: 1016 = I, 1534 = C, and
410 = L.
(XLSX)

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**Author Contributions**

**Conceptualization:** Paula X. Pareja-Loaiza, Liliana Santacoloma Varon, Ronald Maestre-Serrano.

**Data curation:** Paula X. Pareja-Loaiza, Gabriela Rey Vega, Ronald Maestre-Serrano, Audrey Lenhart.

**Formal analysis:** Paula X. Pareja-Loaiza, Gabriela Rey Vega, Ronald Maestre-Serrano, Audrey Lenhart.

**Funding acquisition:** Liliana Santacoloma Varon, Audrey Lenhart.

**Investigation:** Gabriela Rey Vega, Ronald Maestre-Serrano, Audrey Lenhart.

**Methodology:** Paula X. Pareja-Loaiza, Liliana Santacoloma Varon, Ronald Maestre-Serrano.

**Project administration:** Liliana Santacoloma Varon.

**Resources:** Audrey Lenhart.

**Supervision:** Liliana Santacoloma Varon, Audrey Lenhart.

**Validation:** Gabriela Rey Vega, Ronald Maestre-Serrano.

**Writing – original draft:** Paula X. Pareja-Loaiza, Ronald Maestre-Serrano, Audrey Lenhart.

**Writing – review & editing:** Paula X. Pareja-Loaiza, Liliana Santacoloma Varon, Gabriela Rey Vega, Doris Gómez-Camargo, Ronald Maestre-Serrano, Audrey Lenhart.

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