Phenotypic and genotypic resistance to commonly used insecticides in *Aedes aegypti* among four cities in southern Ecuador

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Abstract

Insecticide resistance (IR) can undermine efforts to control vector species of public health importance. *Aedes aegypti* is the main vector of resurging diseases in the Americas such as yellow fever and dengue, as well as recently emerging chikungunya and Zika viruses, which have caused unprecedented epidemics in the region. Vector control remains the primary public health intervention to prevent outbreaks of *Aedes* transmitted diseases. In many high-risk regions, like southern Ecuador, we have limited information on IR in *Ae. aegypti*. In this study, IR status in *Ae. aegypti* was measured across four cities in El Oro Province in Ecuador using phenotypic assays and genetic screening for alleles associated with resistance to pyrethroid insecticides. Bottle bioassays showed significant inter-seasonal variation in resistance to deltamethrin, a pyrethroid insecticide commonly used by the Ministry of Health, and alpha-cypermethrin, as well as differences in deltamethrin resistance between cities. There was also a significant difference in phenotypic response to the organophosphate, Malathion, between two of the cities during the second sampling season. Genotyping showed moderate to high frequencies of the V1016I and F1534C resistance alleles in all four cites. Frequency of resistance genotypes varied significantly between cities in the first sampling season, and not in the later seasons, suggesting a possible selective response to vector control activity. Overall, resistance levels were highest in Machala, a city where dengue is hyperendemic and where insecticide use has historically been more intense than in the other cities included in the study. Despite statistically significant evidence that resistance alleles conferred phenotypic resistance, there was not precise correspondence between these indicators. We found that 17.6% of F1534C and 45.6% of V1016I mutant mosquitoes were susceptible in the bottle bioassays. This study shows there is spatiotemporal variability in IR in southern Ecuador, and serves as an initial examination of the genotypic and phenotypic indicators of IR in this region, providing important information to guide the vector control interventions by the public health sector.

Keywords: Insecticide resistance; *Aedes aegypti*; pyrethroid; organophosphate; Ecuador; arbovirus

Introduction

In Ecuador, dengue, chikungunya, and Zika viruses are present and transmitted to people by the *Aedes aegypti* mosquito, causing a high burden of febrile illness in susceptible populations. This species is a particularly effective vector of these viruses because it has evolved to live in urban environments, lay its eggs in small containers of water in and around human dwellings, and feed preferentially on humans [1]. There were 14,150 cases of dengue reported in Ecuador in the year 2016 [2], while chikungunya caused an estimated 2,025 cases [3] and Zika caused approximately 3,531 cases by the end of 2016 [4]. For all three of these diseases, actual incidence in Ecuador is likely much higher than the reported figures indicate because many cases are asymptomatic or mild, and access to laboratory diagnostics can be limited [5]. Furthermore, co-circulation of these viruses can lead to more severe disease outcomes and differential diagnosis is complicated [6]. To reduce the burden of disease, public health organizations rely heavily on vector control methods, particularly insecticide applications [7]. Additionally, individuals purchase products such as aerosols, repellents, and nets to reduce mosquito populations and prevent disease transmission at the household level, with low-income households in Machala, Ecuador, spending as much a 10% of their discretionary income on these items [5].

There are four main categories of chemical insecticides regularly used for disease vector control: organophosphates, pyrethroids, carbamates, and organochlorines, with organophosphates and pyrethroids being the most widely used in Ecuador for *Ae. aegypti* control [8]. For all four of these insecticide classes, regular deployment in locations around the world has been associated with the development of insecticide resistance (IR) in targeted vector populations, resulting in resurgences of mosquito-borne diseases [9–11]. While public health

organizations have made recommendations for monitoring and managing IR [12], these practices are often resource-intensive, meaning many areas do not have the capacity to conduct regular IR testing [13,14]. Additionally, local-scale IR can be driven by the largely unregulated application of insecticide treatments at the household level by private individuals [15]. When resistance has developed, but remains undetected, public health organizations may unintentionally spend substantial time and resources applying insecticides that are ineffective [16] and may have negative environmental and human health impacts [17].

A better understanding of the spatial and temporal variability in IR would greatly enhance the ability of vector control groups to predict and mitigate IR in vector populations. Research has shown that IR status can differ dramatically across cities within a country [18–20], but further work needs to be conducted to understand the spatial scale of this variability, particularly across cities with differing vector control needs and strategies. Furthermore, most available reports in this area determine IR status at a single point in time, but IR exhibits temporal fluctuations that should be taken into consideration [21,22]. This study addresses a substantial knowledge gap regarding IR in *Ae. aegypti* by investigating differences in IR across both space (with four cities included in the study area) and time (with sampling occurring in three seasons), while also considering both genetic and phenotypic lines of evidence to determine IR status. Furthermore, assays were conducted with multiple commonly-used insecticides, allowing for comparisons of effectiveness that can inform local-scale vector-control decision-making.

In Ecuador, vector control is conducted by field workers of the Ministry of Health (MoH) in areas at risk of arboviral transmission, as well as focal control in and around homes with suspected arboviral infections. Interventions to control adult mosquitoes include indoor residual

spraying (IRS) with deltamethrin (pyrethroid) and ultra-low volume (ULV) fumigation with malathion (organophosphate). Interventions to control juvenile mosquitoes include application of an organophosphate larvicide (temephos) to containers with standing water, as well as community mobilization to eliminate larval habitats.

The objectives of this study were to evaluate overall IR status and describe seasonal and inter-city variability in IR of Ecuadorian populations of *Ae. aegypti*, specifically testing for susceptibility to insecticides commonly used in mosquito control campaigns by the public health sector. In recent work in El Oro province, researchers demonstrated resistance to deltamethrin in phenotypic assays and detected genetic mutations associated with resistance to pyrethroids in *Ae. aegypti* (Neira, *unpublished data*). Beyond this small-scale study, however, IR monitoring is not regularly performed and research in this area has been very limited, meaning the prevalence IR in these *Ae. aegypti* populations is largely unknown. Due to the high morbidity and mortality associated with the viruses transmitted by *Ae. aegypti*, as well as concerns regarding the cost and efficacy of vector control efforts, leaders of the Ministry of Health (MoH) have indicated that there is a critical need for operational research on IR in Ecuador.

Methods

Ethics Statement

This study was part of a longitudinal cohort study. The study protocol was reviewed and approval by Institutional Review Boards (IRBs) at SUNY Upstate Medical University, the Human Research Protection Office (HRPO) of the U.S. Department of Defense, the Luis Vernaza Hospital in Guayaquil, Ecuador, and the Ecuadorean Ministry of Health. Prior to the

start of the study, all adult participants (18 years of age or older) engaged in a written informed consent process.

Data collection for this project was also conducted with input from local vector control and public health organizations. Sampling coordinates were stored in a secured database and all resulting data were pooled to evaluate citywide trends in the analysis, meaning identifiable individual sites were not shared.

Study sites and sampling period

Field samples of *Ae. aegypti* were collected in four cities in El Oro, a province located on Ecuador's southern coast with a well-documented history of dengue transmission (Figure 1). The cities included in this study historically range from high to low dengue case burden. Two sites were located at sea level along the coast – Machala (3°15'09"S, 79°57'20"W; 6m elevation), a port city and important center of agribusiness, and Huaquillas (3°28'33"S, 80°13'33"W; 15m elevation), a town on the border with Peru. These cities have endemic transmission of dengue and typically see high numbers of annual reported cases. In contrast, Portovelo (3°42'58"S, 79°37'08"W; 645 m elevation) and Zaruma (3°41'31"S, 79°36'47"W; 1,155m elevation) are both mining towns located further inland and at higher elevations, and have limited autochthonous transmission. The discrepancies in case burden between the cities translate into differential mosquito control demands at the municipal level, making our selection of study sites a diverse backdrop for investigating IR.

Collection of field samples was conducted in 2017 over three sampling periods: Season 1 (February 1 – April 30), Season 2 (May 1 – June 30), and Season 3 (July 1 – August 31). Seasons were explicitly chosen to collect mosquito eggs at different phases of annual arbovirus

transmission in Ecuador, sampling during the peak (Season 1), decline (Season 2), and low transmission (Season 3). These collection seasons correspond with historical trends in both dengue transmission and mosquito densities in El Oro Province [23,24].

Egg collection and mosquito rearing

Ae. aegypti eggs were collected from households in the four cities using ovitraps lined with oviposition paper [25]. Households were selected from ongoing surveillance study sites where the ministry of health designated areas of the cities as having high historic risk of dengue. These houses are distributed across each of the four cities to capture geographic variability, and are part of a larger study looking at arboviral and vector dynamics in a cohort of homes over 3 years. Ovitrapped houses for this study were purposely targeted to collect the greatest number of eggs, so ovitraps were strategically placed in areas known to have a greater abundance of eggs, representing a subset of the larger household cohort study. Ovitraps are a sensitive means of identifying the presence of *Ae. aegypti*, especially in areas with low vector densities [26]. Papers with eggs were collected in the field and transported to the Center for Research on Health in Latin America (CISeAL) in Quito, Ecuador, where all insect rearing and handling was performed under standard insectary conditions (28 ± 1 ºC, 80% +/- 10% relative humidity, 12h light; 12h darkness photoperiod). Egg hatching was achieved by placing the papers in a plastic tray containing distilled water. Larvae were fed finely ground fish flakes. Upon pupation, mosquito specimens were placed in cages for adult emergence. To increase the number of adult specimens available for experimentation, F0 adults were allowed to mate and adult female mosquitoes were blood fed on mice and allowed to oviposit. Collected eggs (F1 generation) were hatched and maintained under the aforementioned conditions. Upon pupation, F1 specimens were sorted by

sex and females were used for further experimentation. Males were killed by freezing and discarded.

Resistance monitoring

We monitored phenotypic resistance to pesticides using the bottle bioassay method as described by Brogdon and Chan [27]. Briefly, we coated glass bottles with ethanolic-insecticide solutions at established diagnostic doses: 10 µg / bottle for both deltamethrin and alpha-cypermethrin (both pyrethroids), and 50 µg / bottle malathion (an organophosphate). After the ethanol had evaporated from the bottles, groups of 15-25 female mosquitoes were introduced into each bottle and mortality was recorded at 15-minute intervals. Mosquitoes were considered dead when they were incapable of flying or maintaining an upright posture on the surface of the bottle. Final mortality counts were recorded after 30 minutes of exposure. Each bioassay consisted of four pesticide-coated bottles and one control bottle, coated only with the ethanol diluent.

The number of F1 females procured from field-collected specimens allowed us to perform one to three bioassay replicates for each city. The selection of pesticides (deltamethrin, alpha-cypermethrin and malathion) corresponds to pesticides typically used for vector control in Ecuador.

Genotyping

In addition to the monitoring of phenotypic resistance, we also performed genetic screening for two mutations of the voltage-gated sodium channel gene (V1016I and F1534C) that are associated with IR to pyrethroids in *Ae. aegypti*. Both mutations have been previously described

and are reported to alter the transmembrane domain of the voltage-gated sodium channel, preventing pyrethroids from binding to these sites [28,29]. Previous genotyping work has shown that *Aedes aegypti* that are homozygous for the isoleucine variant at position 1016 (I1016) are resistant to pyrethroid treatments, while those that are homozygous for the valine variant (V1016) allele are susceptible, and those that are heterozygous show intermediate resistance [30]. At position 1534, the cysteine variant (C1534) confers resistance, while the phenylalanine variant (F1534) is susceptible, and the heterozygote shows intermediate resistance [31].

Following the aforementioned bioassays, DNA was extracted from specimens of known phenotype (susceptible/resistant to each of the pesticides used for testing). Genomic DNA was obtained using the Wizard ® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA), following the protocol established by the manufacturer. Quantification of the concentration (ng/µL) and purity (absorbance index at 260nm / 280 nm) of the obtained DNA was completed using a Nano-Drop 1000 V3.7 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA).

Screening for the V1016I and F1534C mutations was performed as previously described [28,29], using a Bio-Rad real-time thermocycler CFX96 (Bio-Rad, Hercules, CA, USA). Results were visualized using CFX Manager Software (Version 3.1, Bio-Rad, Hercules, CA, USA). Determination of the genotype was possible through the visualization of PCR product melting curves. In the case of the V1016I mutation, a melting peak at 79ºC corresponded to isoleucine (I/I – resistant mutant) and a melting peak at 85ºC corresponded to a valine (V/V – susceptible wild type) (Figure S1). In the case of the F1534C mutation, a melting peak at 85ºC corresponded to cysteine (C/C, resistant mutant) and a melting peak at 80ºC corresponded to a phenylalanine

(F/F– susceptible wild type) (Figure S2). Genotypic and allelic frequencies were calculated for both mutations.

Seasonal differences in genotypic and phenotypic frequencies for all four cities were mapped using ArcMap© version 10.6 (Environmental Systems Research Institute, Redlands, California, USA) and tested for statistical significance ($\alpha = 0.05$) using Fisher's exact test of independence in the R software for statistical computing, version 3.4.3 (The Foundation for Statistical Computing, Vienna, Austria). When significant differences were detected, post-hoc pairwise Fisher's tests with Bonferroni corrections were then performed to determine which cities differ in the frequency of resistant genotypes. The same statistical methods were used to examine phenotypic frequencies of resistance, as determined by bottle bioassays for deltamethrin, alpha-cypermethrin, and malathion, between cities for each collection season and in relation to corresponding genotypes.

Results

Resistance monitoring

High levels of IR to deltamethrin, alpha-cypermethrin, and malathion were detected in all four cities, as determined by bottle bioassays. Machala had the lowest combined mortality averaged across the three seasons (18.29%, SE±5.75), indicating the highest level of resistance, followed by Portovelo (31.08%, SE±6.23) and Huaquillas (44.79%, SE±10.87). The mean mortality rate for Zaruma was 23.67% (SE±17.93) in Season 1, which was the only time period in which sufficient numbers of *Ae. aegypti* were collected for bioassays at this location.

Significant phenotypic differences in deltamethrin resistance between cities were detected in all collection seasons (Fisher's exact test p-value<0.001) (Figure 2a). This pattern

was also seen in the post-hoc pairwise tests (Table S1). Mean mosquito mortality upon exposure to deltamethrin in bottle bioassays ranged from 0.70% (SE±0.6) in Machala to 18.99% (SE±0) in Huaquillas in Season 1, from 7.09% (SE±4.19) in Portovelo to 16.88% (SE±7.51) in Huaquillas in Season 2, and from 0.37% (SE±0.37) in Machala to 56% (SE±2.49) in Huaquillas in Season 3, with no mortality observed in control groups (Figure 2a).

Mean mosquito mortality upon exposure to alpha-cypermethrin in bottle bioassays ranged from 59.41% (SE±1.49) in Machala to 59.24 (SE±13.86) in Zaruma in Season 1, from 12.1% (SE±0.42) in Machala to 17.27 (SE±0.33) in Portovelo in Season 2, and from 0.38% (SE±0.38) in Machala to 11.39 (SE±1.03) in Portovelo in Season 3, with no mortality observed in control groups (Figure 2b). Significant differences in mean mortality rates between cities were detected in seasons two and three (Fisher's exact test p value<0.001) (Figure 2b), with Machala having the highest proportion of phenotypically resistant specimens in these two seasons (Table S2). Collection counts were insufficient for alpha-cypermethrin bioassays in Portovelo for Season 1, Huaquillas for Seasons 1 and 3, and Zaruma for Seasons 2 and 3.

Mean mosquito mortality when exposed to malathion in the bottle bioassays ranged from 5.07% (SE±0.35) in Machala to 3.03% (SE±1.52) in Zaruma in Season 1, from no mortality in Machala to 1.45% (SE±1.45) in Portovelo in Season 2, and from 0.78% (SE±0.78) in Machala to 2.86% (SE±0.08) in Portovelo in Season 3, with no mortality observed in control groups. Statistically significant differences in Malathion resistance were only detected in season two between Machala and Portovelo (Fisher's exact test p value < 0.001). Due to low sample sizes, bioassays could not be conducted in Portovelo for Season 1, Zaruma for Seasons 2 and 3, and Huaquillas for all three seasons, and therefore further comparisons were not feasible.

Genotyping

Observed frequencies varied significantly between cities only in Season 1 for both V1016I and F1534C alleles (Fisher's exact test p value < 0.001) (Figure 3). Pairwise post-hoc analysis revealed significant differences in the frequencies of genotypes I/I (mutant) and V/I (heterozygous) for the V1061I gene, with Huaquillas having a significantly higher frequency of heterozygotes compared to Machala and Portovelo (Post-hoc Fisher's exact test p value < 0.001 and 0.03, respectively, Table S3). Portovelo and Zaruma differed in the frequency of I/I (mutant) and V/V (wild type) genotypes, with Zaruma having a significantly higher frequency of wild type mosquitoes during Season 1 (Post-hoc Fisher's exact test p value < 0.05, Table S3). Genotypic frequencies of C/C (mutant) and F/C (heterozygous) genotypes of the F1534C resistance gene differed across cities in Season 1, with Zaruma having a significantly higher proportion of the heterozygous genotype than Huaquillas and Machala (Post-hoc Fisher's exact test p value < 0.02 and < 0.001, respectively, Table S4). Although differences in the frequencies of F1534C genotypes also varied significantly in the Season 3, conservative post-hoc analysis did not reveal any significant pairwise relationships (Table S4).

There were statistically significant associations between the resistance genotypes and phenotypic resistance results for 632 *Aedes aegypti* that were genotyped and subjected to the pyrethroid (deltamethrin or alpha-cypermethrin) bottle bioassays. Due to low sample sizes, we pooled pyrethroid assay results (Table 1), and present the separated analyses in supplemental information (Tables S5, S6). For mutation V1016I, while the majority of the resistant individuals (96.4%) presented the mutant or heterozygous genotype, 12 (3.6%) phenotypically resistant individuals presented the wild type (V/V) genotype that is typically associated with susceptibility to pyrethroids, and 40 (13.5%) susceptible individuals presented the genotype that

typically confers resistance. Similarly, at the F1534C locus, 276 (93.2%) individuals with the mutant (C/C) genotype, typically associated with resistance, were susceptible to pyrethroid treatments. Due to low sample sizes of the wildtype (F/F) for both pyrethroids, separate Fisher's exact tests were not conducted (Table S6).

Discussion

In this study, exposure to diagnostic doses of deltamethrin, alpha-cypermethrin and malathion resulted in mortality rates below 80% in all populations tested, regardless of collection season. Based on the World Health Organization´s recommendations for assessing the significance of detected resistance [27], our results suggest that these *Ae. aegypti* populations should be considered as resistant to all the insecticides considered in our study. Furthermore, the results of bioassays for malathion susceptibility are of particular interest, as they indicate that these populations are extremely resistant to malathion, with no population showing more than 5.07% mortality when exposed to this pesticide, and populations from Machala reaching values as low as 0% mortality during Season 2.

The molecular characterization shows that the resistant-associated mutant alleles V1016I and F1534C are present in all the locations studied. In particular, allele F1534C was present at very high frequencies (close to 1) in all the locations studied and across all three seasons, similar to what was seen in recent work in Mexico [32]. This suggests that this gene has been subjected to selective pressures in the past and is approaching or has reached fixation in these populations. Both of these mutations typically confer resistance to dichlorodiphenyltrichloroethane (DDT) as well as pyrethroids [33,34], meaning selection for these alleles likely began with earlier widespread usage of DDT (which was used by the MoH until 1996 [35]) and has persisted as

pyrethroids became more commonly used [36]. Significant distinctions in genotypic and phenotypic frequencies were not detected for many cities in later seasons.

It is worth noting that for some locations and seasons, periods of low vector densities in the field caused the collection of low numbers of mosquito eggs. This scarcity of F0 individuals translated into missing data for some cities and/or very low frequency counts in some categories, thus providing a limited basis for making quantitative comparisons.

The detection of resistance to organophosphate and pyrethroid insecticides in Ecuador is consistent with broader regional trends identified in recent years. In neighboring Peru, *Ae. aegypti* strains have been shown to be resistant to multiple organophosphates and pyrethroids [37], while resistance to commonly used pyrethroids has been detected in Colombia, though the levels of resistance varied throughout the country [19]. However, Colombian populations remain broadly susceptible to malathion, in contrast to the widespread resistance seen in the Ecuadorian *Ae. aegypti* in this study [38]. Throughout the rest of the Americas, pyrethroid resistance appears to be broadly distributed, particularly in Brazil and French Guiana, though some populations in Costa Rica, Panama, and northern Colombia are still susceptible [8].

The temporal and spatial variability in the results from the bioassays highlight the importance of regularly conducting IR monitoring across multiple locations to understand the true extent of IR and make appropriate vector control decisions. For example, in Machala, the mortality rate of *Ae. aegypti* treated with alpha-cypermethrin decreased from season one to season three, indicating this population was becoming less susceptible throughout the course of the study. By contrast, the mortality rate associated with the deltamethrin assays on populations from Huaquillas increased from season one to season three, meaning this population was becoming more susceptible over time. Continued work in this area could determine if these

trends are due to seasonal fluctuations, local-scale movements of *Ae. aegypti* populations with varying levels of resistance, or long-term, inter-annual trends. There were also statistically significant differences in mortality rates across the cities for the deltamethrin bioassays in all three seasons and in two of the three seasons for the cypermethrin bioassays. Considering this variability in the resistance phenotypes found within a single province of Ecuador, organizations involved in decision-making about insecticide applications should be cautioned against inferring the IR status of one *Ae. aegypti* population based on the status of populations in neighboring municipalities.

To better contextualize this work for appropriate vector-control decision-making, the relationships between genotypes, IR bioassay results, and actual IR status in the field should be considered. While the V1016I and F1534C mutations are known markers of pyrethroid resistance in *Ae. aegypti*, the results of this study showed that the genotypes were not perfectly predictive of resistance phenotypes. In the recorded frequencies of genotypes versus bioassay outcomes, resistant and susceptible phenotypes were observed for each genotype, although the resistant phenotype was still statistically associated with the mutant genotypes for both genes. This is likely due to other IR mechanisms, such as metabolic detoxification processes [16], which were not considered in the current study, but could influence IR status in these populations. Additionally, factors such as temperature, larval nutrition, larval density and age have been shown to influence insecticide susceptibility in *Aedes* mosquitoes, leading to discrepancies between bioassay results and the actual outcomes of insecticide treatments in the field [39]. Further work on IR in *Aedes* populations could identify and possibly reconcile differences between results from the laboratory and the field. Additionally, to comprehensively evaluate IR

status, future studies should also investigate the effectiveness of larvicides, such as temephos, which is commonly used in this study area, as well as *Bacillus thuringensis israelensis* (Bti).

Geographic methods, particularly spatial statistics and modelling, are well suited for understanding the patterns and drivers of IR at meso- and local scales. Employing these approaches can lead to more targeted, efficient, and sustainable vector control efforts. Future research in this area should continue to explore the spatial and temporal variability in IR among *Ae. aegypti* populations. Recent work in Yucatan, Mexico, demonstrated that IR levels could vary significantly across neighborhoods within the same city [21]. Additionally, work on the temporal dynamics of resistance could be beneficial for vector control decision-making. For example, in a study on pyrethroid-resistant, field-derived *Ae. aegypti*, researchers demonstrated that susceptibility to pyrethroids could be restored within ten generations when the selective pressure of regular insecticide treatments was removed [22]. While this experiment was conducted in a controlled environment, similar work within the context of urban environments, such as the four cities included in this study, could help calibrate timing of insecticide class rotations, allowing for better long-term management of susceptibility.

In conclusion, the *Ae. aegypti* collected in these four cities in Ecuador showed varying levels of resistance to the insecticides tested, and these measures typically changed over the course of the three seasons during which sampling took place. Regular IR monitoring should be conducted as long as insecticide applications remain an integral component of vector control activities, particularly in areas where these operations are deployed to control arbovirus transmission. Beyond this monitoring process, appropriate alternative management strategies should be deployed when IR is detected. These strategies can include biological control and community mobilization to reduce *Ae. aegypti* breeding sites.

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Figures and Tables

Table 1. Occurrences of V1016I and F1534C Genotypes compared to pyrethroid-resistant phenotype. Fisher's tests indicated significant associations between genotypes and phenotypes for resistance.

Figure 1. Map of the four study site cities: Machala, Huaquillas, Portovelo, and Zaruma, in southern Ecuador. Panel A shows the cities within southern Ecuador, on elevational relief; panel B illustrates Ecuador's location along the northern Pacific coast of South America, and panel C places panel A (red bounding box) within Ecuador's southern coastal region.

Figure 2. Bar plots of CDC bottle bioassay mortality for deltamethrin (A) alpha-cypermethrin (B), and malathion (C). Note different axis height for (C). Asterisks indicate Fisher's exact test showed statistically significant differences across cities for that season. Insufficient numbers of field-collected mosquitos for bioassay within a city in a given season are indicated by 'x'.

Figure 3. Genotypic frequencies of the V1016C and F1534C alleles at the four study site locations during Season 1 (Feburary 1-April 30, 2017).

Supporting information

Table S1: Post-hoc Fisher's exact test p-values for Deltamethrin resistance between cities. Significant difference are denoted in bold.

Table S2: Post-hoc Fisher's exact test *p*-values for Alpha-Cypermethrin resistance between cities in the season two collection period. Significant difference are denoted in bold.

Table S3: Post-hoc Fisher's exact test *p*-values for genotype V1016I in season 1, with comparisons in genotype frequencies made between the cities of Huaquillas (H), Machala (M), Portovelo (P), and Zaruma (Z). Significant difference are denoted in bold.

Table S4: Post-hoc Fisher's exact test *p*-values for genotype F1534C in season 1, with comparisons in genotype frequencies made between the cities of Huaquillas (H), Machala (M), Portovelo (P), and Zaruma (Z). Significant difference are denoted in bold.

Table S5. V1016I and F1534C Genotypes compared to deltamethrin-resistant phenotype

Table S6. V1016I and F1534C Genotypes compared to alpha-cypermethrin resistant phenotype

Figure S1: Genotypic characterization of the V1016I mutation. Dissociation curves representing the susceptible (wild type) homozygous (A), heterozygous (B), and resistant (mutant) heterozygous (C) genotypes.

Figure S2: Genotypic characterization of the F1534C mutation. Dissociation curve representing the wild type (susceptible) homozygous (A), heterozygous (B), and mutant (C) genotypes.

Table 1.

V1016I		**I/I (mutant)**	**V/I (heterozygote)**	**V/V (wild type)**	***p*-value***
	Resistant	187	137	12	**<0.001***
	Susceptible	40	172	84	
F1534C		**C/C (mutant)**	**F/C (heterozygote)**	**F/F (wild type)**	
	Resistant	328	7	1	**0.02***
	Susceptible	276	17	3	

* Fisher's Exact Test

Figure 1

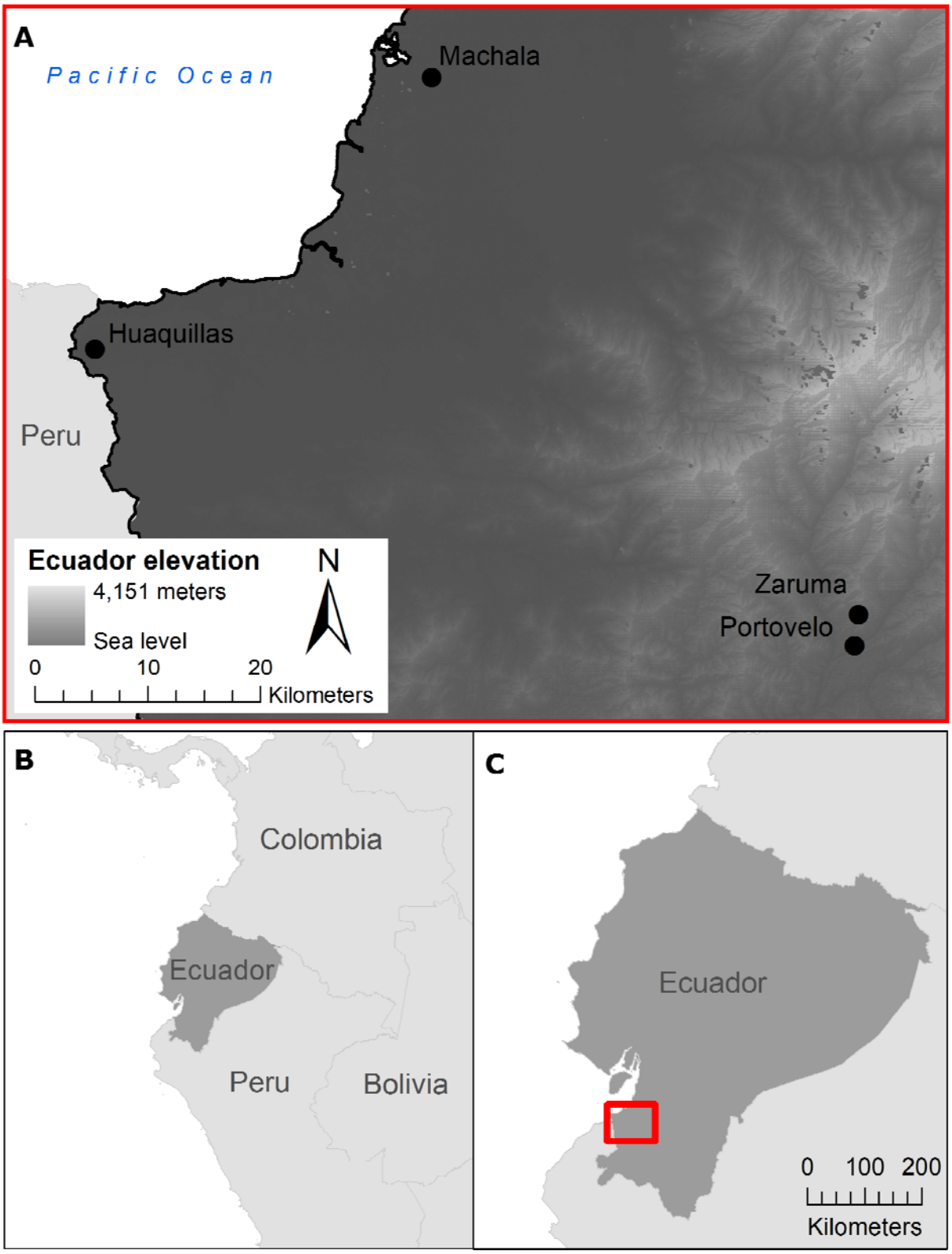

A
Pacific Ocean
Machala
Huaquillas
Peru
Ecuador elevation
4,151 meters
Sea level
N
0
10
20
Kilometers
Zaruma
Portovelo
B
Colombia
Ecuador
Peru
Bolivia
C
Ecuador
0
100
200
Kilometers

Figure 2

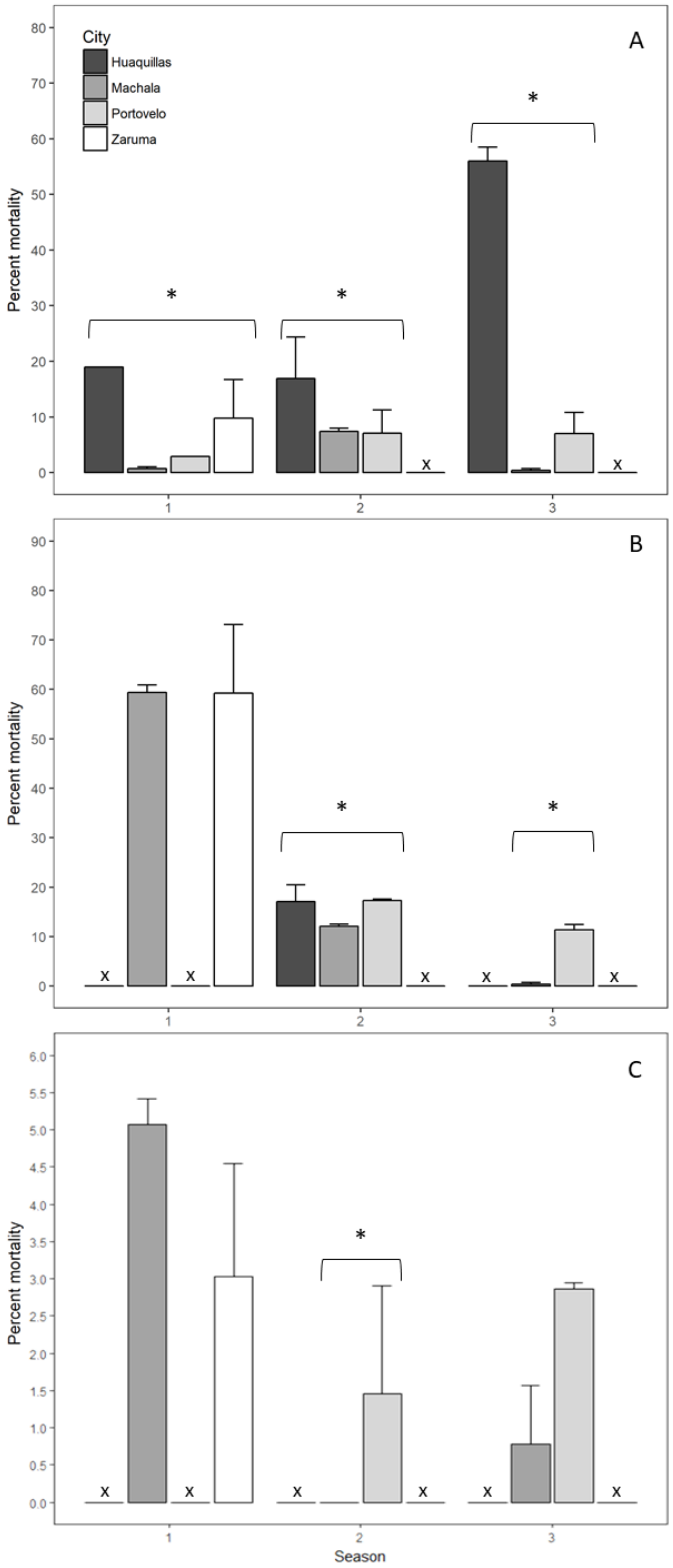

City
Huaquillas
Machala
Portovelo
Zaruma
A
B
C
Percent mortality
Season
1
2
3

Figure 3

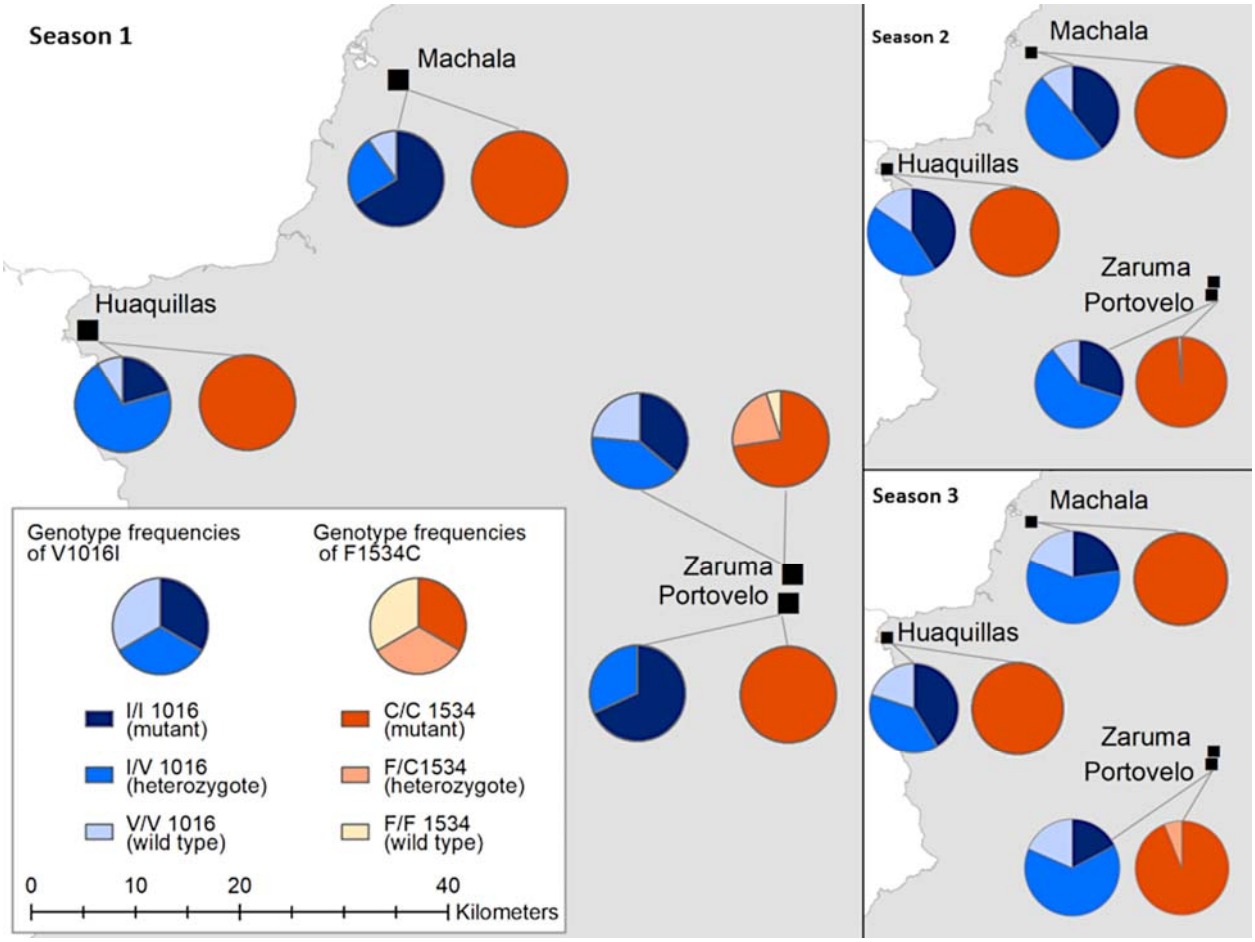

Season 1
Machala
Huaquillas
Zaruma
Portovelo
Genotype frequencies of V1016I
Genotype frequencies of F1534C
I/I 1016 (mutant)
I/V 1016 (heterozygote)
V/V 1016 (wild type)
C/C 1534 (mutant)
F/C1534 (heterozygote)
F/F 1534 (wild type)
0
10
20
40
Kilometers
Season 2
Machala
Huaquillas
Zaruma
Portovelo
Season 3
Machala
Huaquillas
Zaruma
Portovelo

Table S1.

Season	City	Huaquillas	Machala	Portovelo
1	Machala	**< 0.001***		
	Portovelo	**0.002***	1	
	Zaruma	**0.008***	**< 0.001***	0.75
2	Machala	**0.006***		
	Portovelo	**< 0.001***	1	
3	Machala	**< 0.001***		
	Portovelo	**< 0.001***	**< 0.001***	

Table S2.

City	Huaquillas	Machala
Machala	**0.01***	
Portovelo	**< 0.001***	1

Table S3.

City:City*	I/I : V/I	I/I : V/V	V/I : V/V
H:M	**< 0.001***	1.00	1.00
H:P	**0.03***	0.94	1.00
H:Z	0.44	1.00	0.32
M:P	1.00	1.00	1.00
M:Z	0.09	0.08	1.00
P:Z	1.00	**0.05***	1.00

*H – Huaquillas, M-Machala, P-Portovelo, Z-Zaruma

Table S4.

City:City*	C/C : F/C	C/C : F/F	F/C : F/F
H:M	1.00	1.00	1.00
H:P	1.00	1.00	1.00
H:Z	**0.02***	1.00	1.00
M:P	1.00	1.00	1.00
M:Z	**< 0.001***	1.00	1.00
P:Z	0.18	1.00	1.00

*H – Huaquillas, M-Machala, P-Portovelo, Z-Zaruma

Table S5.

V1016I		**I/I (mutant)**	**V/I (heterozygote)**	**V/V (wild type)**	***p*-value***
	Resistant	107	79	10	**<0.001***
	Susceptible	17	88	52	
F1534C		**C/C (mutant)**	**F/C (heterozygote)**	**F/F (wild type)**	
	Resistant	190	5	1	0.10
	Susceptible	144	10	3	

*Fisher's Exact Test

Table S6.

V1016I		**I/I (mutant)**	**V/I (heterozygote)**	**V/V (wild type)**	***p*-value***
	Resistant	80	58	2	**<0.001***
	Susceptible	23	84	32	
F1534C		**C/C (mutant)**	**F/C (heterozygote)**	**F/F (wild type)**	
	Resistant	138	2	0	0.10
	Susceptible	132	7	0	

*Fisher's Exact Test

Figure S1

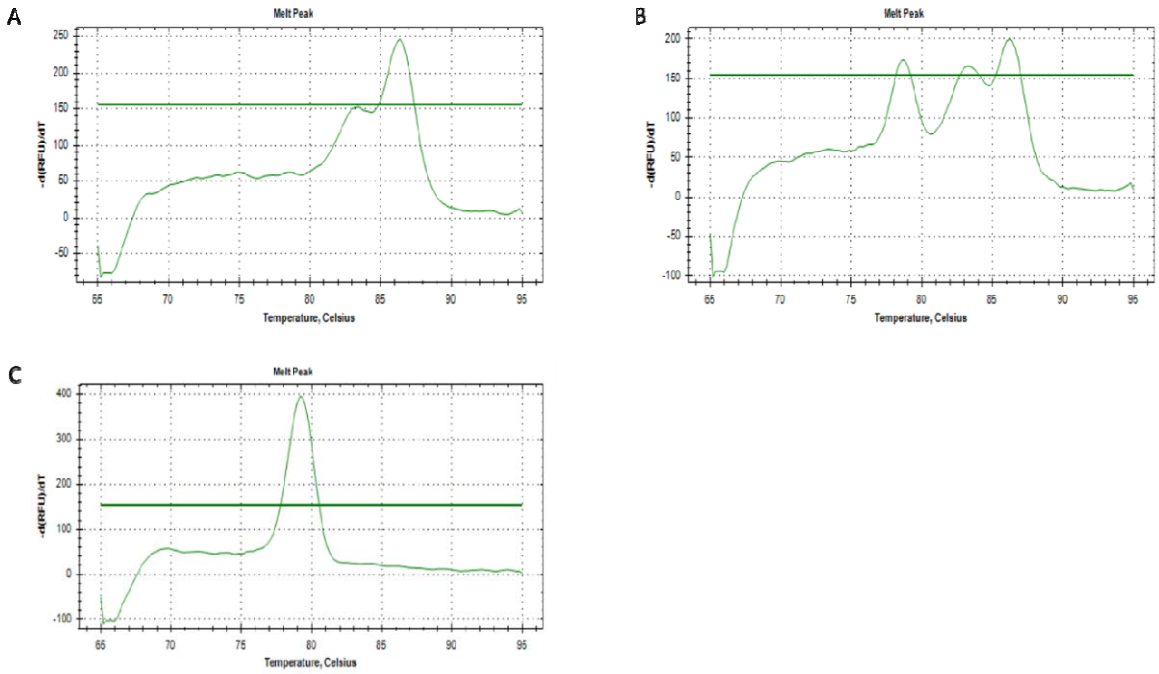
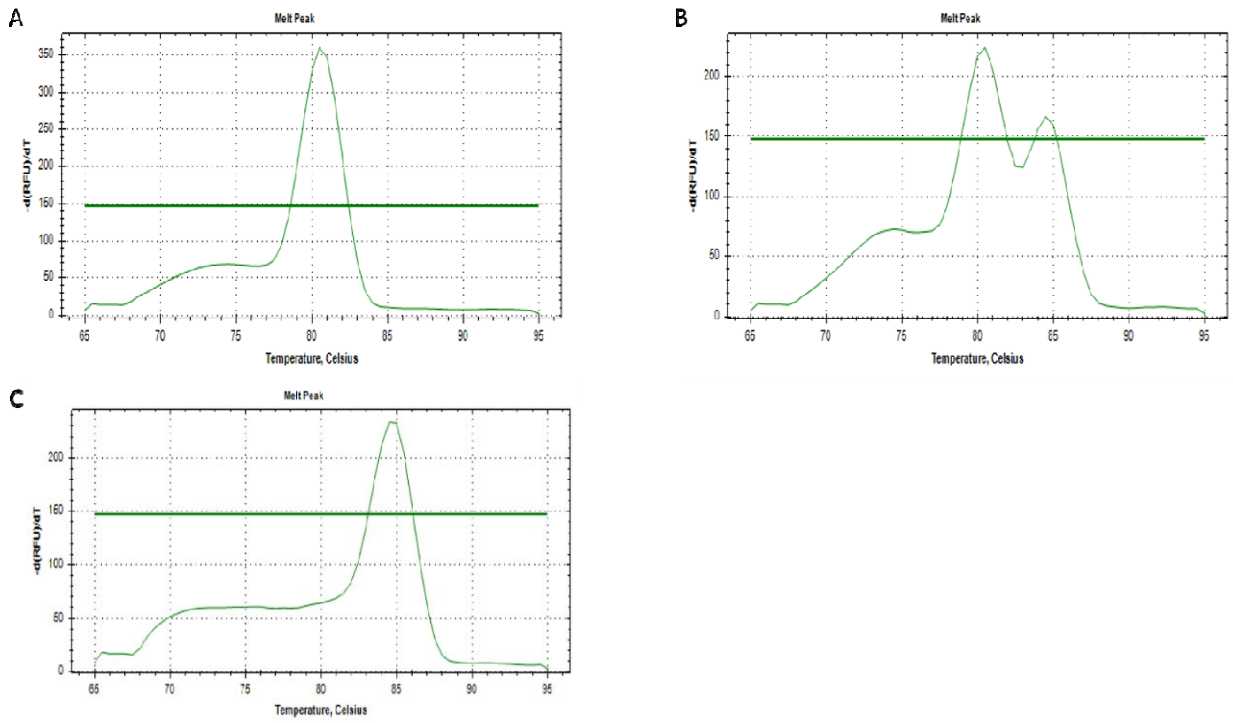

A
Melt Peak
-d(RFU)/dT
Temperature, Celsius
B
Melt Peak
-d(RFU)/dT
Temperature, Celsius
C
Melt Peak
-d(RFU)/dT
Temperature, Celsius

Figure S2


A
Melt Peak
-d(RFU)/dT
Temperature, Celsius
B
Melt Peak
-d(RFU)/dT
Temperature, Celsius
C
Melt Peak
-d(RFU)/dT
Temperature, Celsius