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Multi-Year Mass-Trapping With Autocidal Gravid Ovitraps has Limited Influence on Insecticide Susceptibility in *Aedes aegypti* (Diptera: Culicidae) From Puerto Rico

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Abstract

Mass-trapping has been used to control outbreaks of *Aedes aegypti* (Linnaeus) (Diptera: Culicidae) in Puerto Rico since 2011. We investigated the effect of multi-year, insecticide-free mass trapping had on the insecticide susceptibility profile of *Ae. aegypti*. Eggs collected in southern Puerto Rico were used to generate F_1 populations that were tested for susceptibility to permethrin, sumethrin, bifenthrin, deltamethrin, and malathion according to CDC bottle bioassays protocols. All populations of *Ae. aegypti* were resistant to the synthetic pyrethroids and mosquitoes from two locations were partially resistant to malathion. Population genetic analysis, using a double digest restriction sites associated DNA sequencing (ddRADseq) approach, indicated a large amount of migration between study sites effectively homogenizing the mosquito populations. Mass-trapping using noninsecticidal autocidal gravid ovitraps did not restore susceptibility to five active ingredients that are found in commercial insecticides. Migration between communities was high and would have brought outside alleles, including resistant alleles to the treatment communities. Further investigation suggests that household use of commercially available insecticide products may continue to select for resistance in absence of public health space spraying of insecticides.

Key words: *Aedes aegypti*, insecticide resistance, population genetics, Puerto Rico

Insecticide resistance to first-in-line insecticides is a growing problem throughout the Caribbean (Guedes et al. 2020). In Puerto Rico DDT resistance was first reported in the 1960s and resistance to organophosphates and pyrethroids was detected in the 80s (Fox and Garcia-Moll 1961, Flynn et al. 1964, Fox 1980, Hemingway et al. 1989). Recent testing in Puerto Rico found widespread resistance to nearly all synthetic pyrethroids and organophosphates (Ponce-Garcia et al. 2016, Estep et al. 2017, Hemme et al. 2019). In order to prevent or slow the evolution of insecticide resistance, it has been recommended that mosquito control programs rotate insecticides annually, target and eradicate focal populations of resistant mosquitoes with insecticides with a different mode of action or employ mosaic spray treatments (Curtis 1981). These strategies are intended to dilute the proportion of resistant alleles in the population with those that are susceptible to insecticides, thus limiting the spread of alleles that confer resistance. However, evidence is sparse and limited to laboratory studies while field-based studies indicate the opposite, perhaps due to partial cross-resistance to multiple insecticides (Melo-Santos et al. 2010, Macoris et al. 2018). In Brazil, 10 yr after vector control agencies ceased using pyrethroids to control *Aedes aegypti* (Linnaeus) (Diptera: Culicidae), it remained resistant (Macoris et al. 2018). It was reported that the use of pyrethroids for domestic and household use was selected for resistance and later empirical evidence

from Mexico confirmed the link between household use of insecticides and resistance in *Ae. aegypti* (Gray et al. 2018, Macoris et al. 2018).

CDC autocidal gravid ovitraps (AGO) have been used to control outbreaks of *Ae. aegypti* in the city of Caguas, Puerto Rico and the communities of La Margarita and Villodas in the municipality of Salinas, Puerto Rico (Barrera et al. 2014a, b, 2019a). In Caguas, trapping reduced the abundance of female *Ae. aegypti* by 82% for nine months, removing an estimated 6 million female *Ae. aegypti* from the environment (Barrera et al. 2019b). Similar observations were reported in La Margarita and Villodas, where traps reduced the population of female *Ae. aegypti* by >80% (Barrera et al. 2014a, b, Lega et al. 2020). Mass-trapping reduces the risk of arbovirus transmission by suppressing the vector population to levels that are unable to support continued transmission (Barrera et al. 2019a, Sharp et al. 2019).

Aedes aegypti from Salinas and Guayama were resistant to simple pyrethroids and a large proportion of mosquitoes contained knockdown resistant (*kdr*) mutations when genotyped (Ponce-Garcia et al. 2016, Hemme et al. 2019). As *kdr* mutations (Ile1016 and Cyc1534) were not fixed within the populations a proportion remained susceptible to pyrethroids. Wild, pyrethroid-resistant *Aedes aegypti* reared in the laboratory revert to being susceptible within eight generations in the absence of insecticide exposure (Vera-Maloof et al. 2020). Here, we hypothesized that multi-year suppression (8 yr in La Margarita and 6 yr in Villodas) of *Ae. aegypti* using noninsecticidal AGO traps reduce household use of insecticides and in the absence of local and municipal adulticiding programs would remove selective pressures that maintained the insecticide-resistant phenotype in those populations. Furthermore, the proportion of mosquitoes with the susceptible phenotype would increase in part because of fitness costs associated with insecticide resistance (Brito et al. 2013, Ponce-Garcia et al. 2016, David et al. 2018, Saingamsook et al. 2019). In this study, we investigated whether mass-trapping affected the insecticide susceptibility of *Ae. aegypti*.

Materials and Methods

Study Site

This study was conducted in communities located in the southern municipalities of Salinas and Guayama, Puerto Rico (Fig. 1). Demographic data from the treatment and nontreatment control sites were examined using census data from the 2018 American Community Survey (Supp. Table S1 [online only]). The effectiveness of mass-trapping on *Ae. aegypti* abundance and arbovirus transmission within these communities has been extensively investigated in previous studies (Barrera et al. 2014a, b, 2017, 2019a, Lorenzi et al. 2016). Briefly, the density of *Ae. aegypti* were compared in the treatment communities of La Margarita and Villodas and the nontreatment control communities of Arboleda and Playa (Barrera et al. 2014a, b). Three CDC Autocidal Gravid Ovitraps (AGO) per house were deployed at more than 80% of the households in the treatment communities and traps were deployed in 2011 and 2013 in La Margarita and Villodas, respectively and continuously controlled until 2019. Populations of *Ae. aegypti* were significantly reduced by 79% in the treatment sites (Barrera et al. 2014b). Neither mosquito control AGOs nor Sentinel-AGOs were in operation in the communities of Pueblo or Villa Esperanza.

Ae. aegypti Egg Collections for Insecticide Resistance Testing

Aedes aegypti eggs were collected in Arboleda, La Margarita, Playa, and Villodas to generate F_1 populations to test for insecticide susceptibility and determine the effects of mass-trapping on susceptibility (Fig. 1). From January to February 2019, 120 ovicups with a hay solution of 10% and 100% were deployed in all four communities as described in Reiter et al. (1991). Ovicups were deployed weekly for three consecutive days over four weeks to maximize the number of eggs collected. In the nontreatment control sites 60% of the egg papers were positive while in the treatment sites 30% of the egg papers were positive for *Ae. aegypti* eggs. Egg papers were brought back to the laboratory at the CDC Dengue Branch and reared using

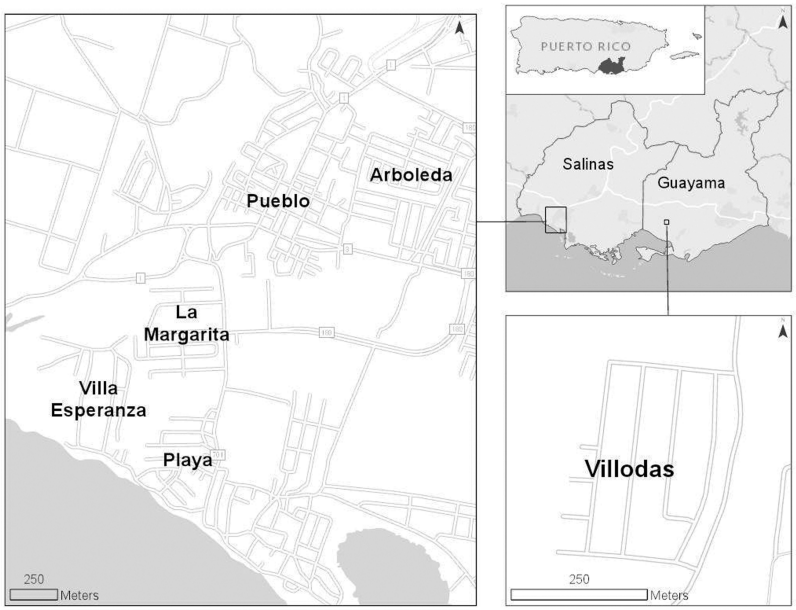

Fig. 1. Location of *Aedes aegypti* egg and adult collections. Communities where autocidal gravid ovitraps controlled outbreaks of *Aedes aegypti* (La Margarita and Villodas) and communities where *Aedes aegypti* were collected for genetic analysis (La Margarita, Playa, Pueblo, Villa Esperanza, and Villodas) and susceptibility to insecticides (Arboleda, La Margarita, Playa, and Villodas).

standard laboratory protocols (Hemme et al. 2016). As not enough eggs and subsequently adults were collected from the treatment sites to test for resistance to insecticides, four F_1 mosquito populations were generated from the eggs that were collected in Arboleda, La Margarita, Playa, and Villodas. Egg papers containing F_1 eggs were stored for one month until bottle bioassays were conducted at which point, they were conditioned in water and reared to adults (Hemme et al. 2016).

Insecticide Susceptibility Testing

Female F_1 *Ae. aegypti* (2–3 d old) from La Margarita, Villodas, Playa, and Arboleda were tested for susceptibility to four synthetic pyrethroid insecticides (permethrin, sumethrin, bifenthrin, deltamethrin) and one organophosphate (malathion) using the CDC bottle bioassay kit (CDC 2019). Briefly, technical grade active ingredients (AIs) were purchased from ChemService (West Chester, PA), diluted in acetone to 43, 20, 15, 0.75, and 400 µg/ml according to CDC recommendations (CDC 2019, Hemme et al. 2019). Diluted AIs were evenly coated on 250 ml Wheaton glass bottles (DWK Life Sciences, Millville, NJ) and placed uncapped on bottle rollers until dry. Control bottles containing only acetone were prepared in the same manner.

*Ae. aegypt*i Rockefeller were used as insecticide susceptible controls to confirm bottles were coated appropriately. To test for resistance, 19–26 female *Ae. aegypti* were introduced into four AI-treated bottles and one acetone control bottle. Mortality was recorded at 10 min intervals for permethrin and sumethrin and at 15 min intervals for bifenthrin, deltamethrin, and malathion. Resistance testing at Arboleda, La Margarita, and Playa were stopped at 120 min while testing at Villodas was stopped at 60 min. Resistance was determined using CDC-recommended diagnostic times at 10, 10, 30, 30, and 15 min for permethrin, sumethrin, bifenthrin, deltamethrin, and malathion. Mosquitoes were scored as being dead if it could not stand or fly. Insecticide resistance, partial resistance, and susceptibility for mosquito populations were interpreted from bioassay data at <90, 90–98, and >98% mortality, respectively according to WHO recommendations (WHO, 2013).

Adult *Ae. aegypti* Collections for Genetic Analysis

Genetic structure and migration into the mass-trapping treatment community of La Margarita was estimated using a random sample of adult *Ae. aegypti* from La Margarita, Playa, Pueblo, Villa Esperanza, and San Juan (Fig. 1). Mosquitoes were collected from the field in November 2014 using BioGents (BG) Sentinel traps with black coverings and without lures and remained in storage at −80°C at Dengue Branch facilities. A sub-sample of 20 *Ae. aegypti* females from each community were selected for genotyping from 48 BG traps in La Margarita and from 12 BG traps in Playa, Pueblo, Villa Esperanza, and San Juan.

Genetic Markers and ddRADseq Library Construction

To facilitate population genetic analyses, double-digest restriction-associated DNA sequencing (ddRADseq) was employed, allowing for sequencing of the same ~1% of the genome from every mosquito collected. This reduced-representation library preparation method facilitates genetic profiling of hundreds of mosquitoes across multiple geographic populations at hundreds of thousands of genomic sites (see results for exact numbers) without the need for costly whole-genome sequencing. Single Nucleotide Polymorphisms (SNPs) were identified using ddRADseq libraries constructed following Turissini et al. (2014). DNA was extracted from adults using the ZR-96 Quick-gDNA kit (Zymo Research; Irvine, CA) and was digested for three hours at 37°C with *MluC1* and *NlaIII* (New England Biolabs [NEB]; Ipswich, MA). Digested DNA was purified using homemade "Ampure" magnetic beads. A barcoded adapter (1 of 48) was then ligated to the sticky end produced by *NlaIII*, and a universal adapter was ligated to the sticky end produced by *MluC1* using T4 DNA ligase (NEB). Reactions from uniquely barcoded individuals were then pooled together, concentrated, and cleaned using two rounds of magnetic bead purification. These sub-libraries were size selected on a Blue Pippin (Sage Science; Beverly, MA) on a 1.5% gel cassette with the "tight" setting and a 400 bp fragment target. Size-selected DNA from each sub-library was then PCR amplified using one of 12 indexed primers. Each PCR reaction consisted of 1x NEB Q5 PCR buffer, 10mM each DNTP, 20 pmol of the universal and indexed primer, 0.25 U of NEB Q5 DNA polymerase, and 4 µl of template. The reactions were carried out as follows: 98°C for 1 min; 12 cycles of 98°C for 8 s, 68°C for 20 s, and 72°C for 20 s; and a final extension at 72°C for 2 min. Following the PCR reaction, there were 576 (12 indexed primers × 48 barcodes) uniquely barcoded/indexed individuals that could be multiplexed for sequencing. This was done by first checking the quality and quantity of each of the twelve sub-libraries on a BioAnalyzer 2100 (Agilent Technologies; Santa Clara, CA), after which equal molar amounts of each sub-library were pooled to create a final sequencing library. This library was then sequenced on three lanes of the Illumina HiSeq 2500 (Illumina; San Diego, CA) at the UCR genomics core using 100 bp paired-end reads.

Read Mapping and SNP Calling

After library construction and replicate sequencing runs on three lanes of the Illumina HiSeq 2500, samples were demultiplexed and reads were filtered and trimmed for quality using custom scripts. Reads were then mapped to the *Ae. aegypti* reference genome using the Burrows–Wheeler aligner (BWA, Li and Durbin 2009) BWA-MEM algorithm with default settings. SNPs were called on a per population basis using the Genome Analysis ToolKit HaplotypeCaller algorithm with default settings (DePristo et al. 2011).

Analysis of Genetic Structure

Population structure and whether each community represented a unique genetic population were tested using STRUCTURE (Supp. Fig. S1 [online only]) (Pritchard et al. 2000). We subsampled our set of SNPs so that only sites with data for at least 75% of individuals were included. STRUCTURE estimates the number of subpopulation (K) within a total population. STRUCTURE was run for K = 1–10 on each of 10 different random samples of 20,000 SNPs from this filtered set. After an initial burn-in of 100,000 replicates, we ran 100,000 analysis replicates for each K and determined the best fitting K value for each random sample of SNPs by calculating the relative probability, $\ln Pr(X|K)$, of each K.

Results

Demographic Description of Study Sites

Median property value ranged from $57,700 to $93,000 and was highest in Arboleda and La Margarita. Over the prior 12 mo from when the survey was completed, Playa had the lowest median household income at $8,365, followed by La Margarita ($16,333), Villodas ($20,545) and Arboleda ($26,308). Poverty levels were highest in Playa with 73% below the poverty line while 60, 56, and 39% of households in Villodas, La Margarita, and Arboleda were below the poverty line. In general, the communities of Arboleda

(nontreatment control) and La Margarita (treatment) had a higher socio-economic status than Playa and Villodas: higher median property values, higher proportion of residents with high school diplomas, and lower levels of poverty.

Insecticide Susceptibility of *Ae. aegypti*

All populations tested were resistant to the pyrethroids and showed strong mechanisms of resistance, contradicting the original hypothesis that mass-trapping removed selective pressures that favored mosquitoes with the insecticide resistance phenotype (Table 1 and Fig. 2A–E). We observed a mortality rate of 11% in the Villodas population, 3% mortality in Playa, and 0% mortality in Arboleda and La Margarita (Table 1). Similar rates of mortality were observed when mosquitoes were challenged with sumethrin where 5, 3, 2, and 0% of mosquitoes died from in the Viloodas, Arboleda, Playa, and La Margartia, respectively (Table 1). All populations were resistant to bifenthrin; however, the highest mortality rate was observed in Villodas (46%), followed by Playa (39%), Arboleda (15%), and La Margarita (12%) (Table 1). Mortality rates of 10, 6, 3, and 0% were recorded in Villodas, La Margarita, Playa, and Arboleda when challenged with deltamethrin. The most effective insecticide was malathion. In Arboleda and La Margarita more than 90% of the mosquitoes died within the 15 min diagnostic time and 64% and 23% of the mosquitoes from Villodas and Playa died within 15 min; however, 100% of the mosquitoes died within the margin of error prior to the 30 min time point, suggesting that the populations from the four sites were are susceptible to malathion (Fig. 2A–E).

Read Mapping and SNP Calling

A total of 568,989,132 reads across three lanes of sequencing passed QC filters and were successfully assigned to individuals (~87%), based on barcode and index sequences, allowing for up to one mismatch per barcode and index. This resulted in an average of 989,546 (median: 525,969) reads per individual. The Villodas population had a total of 5,187,366 polymorphic sites with individuals having data for, on average, 526,706 sites (median: 352,342). The samples obtained from La Margarita had 5,518,965 total polymorphic sites and an average of 619,909 (median: 414,758) sites with data per individual. Mosquitoes from Playa, Pueblo, and Villa Esperanza were used as controls and had a total of 1,778,915 polymorphic sites and an average of 313,659 (median: 248,845) sites with data per individual.

Analysis of the Genetic Structure

For all runs, the most appropriate k estimated by STRUCTURE was 2 (relative $\ln Pr(X|K)$ for $k = 2$ was >0.99 for all replicates, Supp. Fig. S1 [online only]). Individual mosquitoes from La Margarita, Playa, Pueblo, Villa Esperanza, and San Juan were grouped into a single genetic cluster, with a small number of individuals from each community showing a small fraction (maximum 3.8%) of their genome belonging to a secondary cluster; individuals without a secondary fraction of their genome account for 52% of all samples. This suggested a high degree of migration and admixture between these five communities. When the estimated subpopulations (k) was increased to 5, which would be expected to group individuals by their sampling site if there were genetic population structure between communities, all individuals from the communities sampled have >95% of their genome belonging to the first genetic cluster, with a few individuals showing a small fraction (maximum 3.9%) of their genome belonging to secondary clusters (Supp. Fig. S1 [online only]). In total, 45% of individuals in this scenario showed no trace of a secondary genetic cluster, while 31% of individuals showed traces of multiple secondary clusters. We also tested to see whether we could detect genetic structure between samples collected in La Margarita and Villodas. We ran STRUCTURE on the adult samples using 10 random samples of 10,000 SNPs, STRUCTURE was run with settings at $k = 1$–10 and for all replicates the most likely k was 1 (relative $\ln Pr(X|K)$ for $k = 1$ was >0.999 for all replicates), meaning that these two communities comprise a single genetic population.

Discussion

As part of insecticide resistance surveillance activities in Puerto Rico we investigated whether multi-year control of *Ae. aegypti* with AGO mass-trapping influences the profile of insecticide susceptibility within these populations. We reasoned that because the burden and abundance of mosquitoes were suppressed for at least 6 yr (8 yr in La Margarita) there was reduced need to apply insecticides, thereby no longer selecting for the resistance phenotype. Findings from this study do not support our original hypothesis. *Aedes aegypti* from the nontreatment control communities were not more sensitive to insecticides. Results from the bottle assays showed that all populations were resistant to synthetic pyrethroids at diagnostic times and doses. We also found that *Ae. aegypti* from the untreated site of Playa and treated sited site of Villodas were resistant to malathion, while mosquitoes from the untreated site of Arboleda were partially resistant to malathion.

One explanation for our findings is that mosquitoes were moving in and out of the study sites which is supported by the STRUCTURE analysis (Supp. Fig. S1 [online only]). We detected high amounts of homogenization between populations, independent of geographic distance and little to no population substructure. High rates of migration between the treated study sites and the surrounding untreated communities would explain the observed patterns of populations homogenization. More importantly, migration would also bring insecticide-resistant alleles into the treatment sites, preserving the resistance phenotype. Alternatively, the homogenization we detected could be because our ddRADseq protocol was not optimized for *Aedes*. However, the STRUCTURE results corroborate recent findings indicating that *Ae. aegypti* populations in the Americas

Table 1. Percent mortality of *Aedes aegypti* collected in the study sites in southern Puerto Rico after exposure to five active ingredients (permethrin, sumethrin, bifenthrin, deltamethrin, malathion) found in insecticides at diagnotic doses and times

Community	Permethrin 43 µg at 10 min	Sumethrin 20 µg at 10 min	Bifenthrin 15 µg at 30 min	Deltamethrin 0.75 µg at 30 min	Malathion 400 µg at 15 min
Arboleda	0.0	3.0	15.2	0.0	92.8
La Margartita	0.0	0.0	12.2	5.7	91.3
Playa	3.0	2.0	38.8	3.2	23.2
Villodas	10.9	5.1	46.2	10.2	64.4

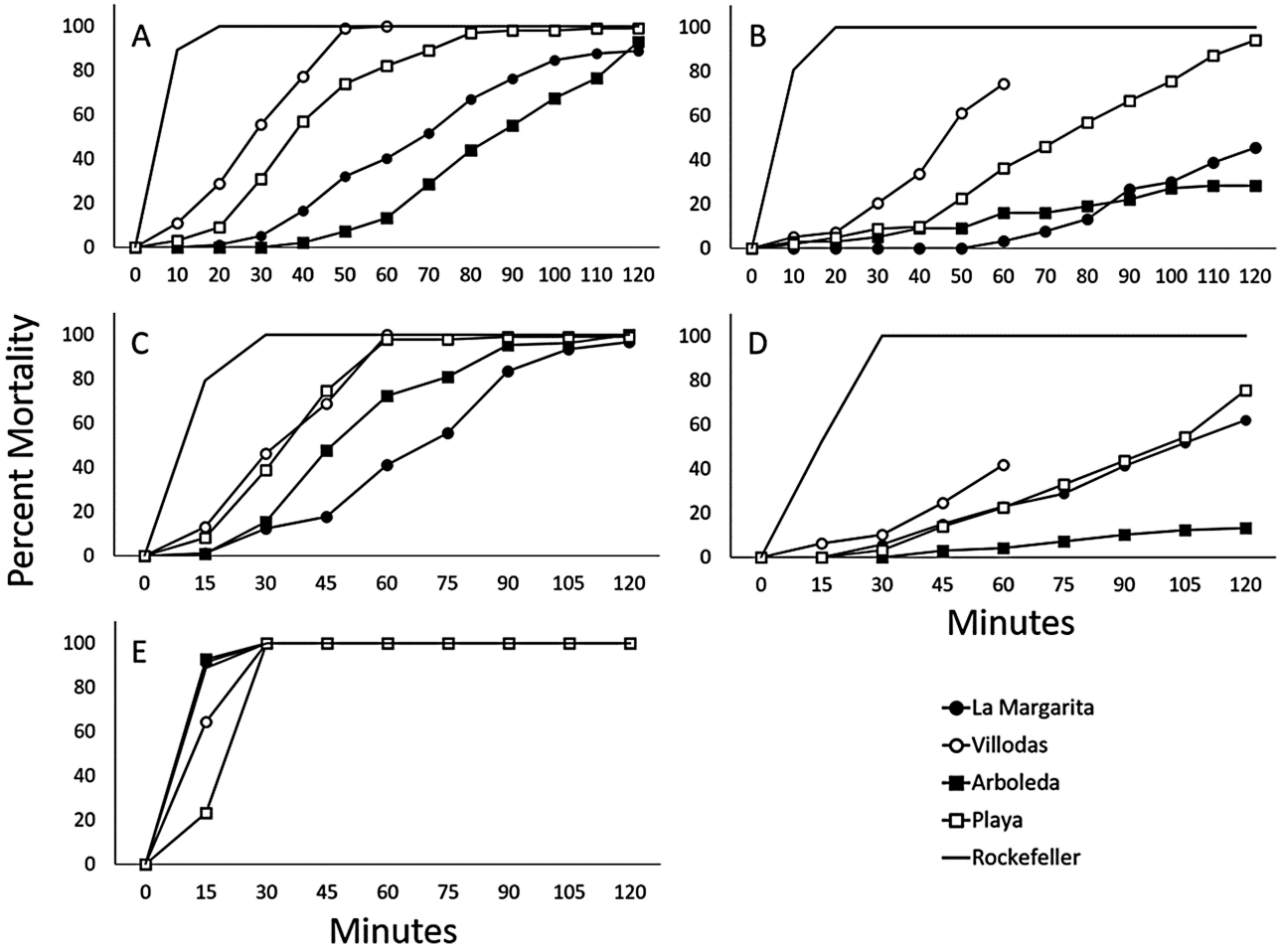

Fig. 2. Mortality of female *Aedes aegypti* collected in southern Puerto Rico to permethrin (A), sumethrin (B), bifenthrin (C), deltamethrin (D), and malathion (E) over 120 min using diagnostic doses of 43, 20, 15, 0.75, and 400 μg for permethrin, sumethrin, bifenthrin, deltamethrin, and malathion.

likely arose out of a very small founding event and as such, spatial population differentiation is difficult to detect (Crawford et al. 2017).

A second, but not mutually exclusive explanation is that homeowners continued to purchase and apply commercial insecticide products for other pests, including non-*Ae. aegypti* nuisance mosquitoes, either themselves or through pest control companies. In Brazil, household use of commercial insecticide products was believed to select for pyrethroids resistance even when it was discontinued for public health purposes (Macoris et al. 2018). While in Mexico, surveys found that >80% of households use pyrethroid-based commercially available insecticides contributed to maintenance of insecticide resistance in *Ae. aegypti* (Gray et al. 2018). If the proportion of households that use commercially available pyrethroid-based insecticides in Puerto Rico is similar to that in Mexico, it would likely have a similar impact on *Ae. aegypti* populations and help explain the rates of insecticide resistance we saw in mosquitoes collected from southern Puerto Rico.

We found that mosquitoes collected in Arboleda and La Margarita, had consistently stronger mechanisms of resistance to pyrethroids than mosquitoes collected in Playa and Villodas. Residents from Arboleda and La Margarita benefited from a higher socio-economic status (SES). Median property values were higher while rates of poverty and percent of residents without a high school diploma were lower in Arboleda and La Margarita. These data suggest that SES and disposable income may be linked with or correlate with household use of commercially available insecticides and maintenance of insecticide resistance in *Ae. aegypti*. Our next steps are to investigate if household use of commercially available insecticides does indeed contribute to the evolution of resistance in *Ae. aegypti* and if socio-economic status and disposable income might modulate resistance.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

Acknowledgments

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