Pyrethroid susceptibility reversal in *Aedes aegypti:* a longitudinal study in Tapachula, Mexico

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Short title: Pyrethroid resistance reversal in field *Aedes aegypti*

Abstract

Pyrethroid resistance in *Aedes aegypti* has become widespread after almost two decades of frequent applications to reduce the transmission of mosquito-borne diseases. Because few insecticide classes are available for public health use, insecticide resistance management (IRM) is proposed as a strategy to retain their use. A key hypothesis of IRM assumes that negative fitness is associated with resistance, and when insecticides are removed from use, susceptibility is restored. In Tapachula, Mexico, pyrethroids (PYRs) were used exclusively by dengue control programs for 15 years, thereby contributing to selection for high PYR resistance in mosquitoes and failure in dengue control. In 2013, PYRs were replaced by organophosphates—insecticides from a class with a different mode of action. To test the hypothesis that PYR resistance is reversed in the absence of PYRs, we monitored *Ae. aegypti's* PYR resistance from 2016 to 2021 in Tapachula. We observed significant declining rates in the lethal concentration 50 (LC_{50}), for permethrin and deltamethrin. For each month following the discontinuation of PYR use by vector control programs, we observed increases in the odds of mosquitoes dying by 1.5% and 8.4% for permethrin and deltamethrin, respectively. Also, knockdown-resistance mutations (*kdr*) in the voltage-gated sodium channel explained the variation in the permethrin LC_{50}s, whereas variation in the deltamethrin LC_{50}s was only explained by time. This trend was rapidly offset by application of a mixture of neonicotinoid and PYRs by vector control programs. Our results suggest that IRM strategies can be used to reverse PYR resistance in *Ae. aegypti*; however, long-term commitment by operational and community programs will be required for success.

Author summary

The mosquito *Aedes aegypti* is the principal urban vector of the viruses that cause three globally significant diseases: dengue fever, chikungunya, and Zika fever, for which vaccines and effective treatments are currently absent. The only way to control dengue, chikungunya, and Zika fever outbreaks is to diminish vector populations. During epidemics, the most frequent way of targeting adult mosquitoes is outdoor spatial spraying of insecticides. Control of *Ae. aegypti* is difficult because limited insecticide classes are available for public health, leading to operational practices that overuse single molecules for long periods of time, imposing great selection pressure on mosquito populations for insecticide resistance. Insecticide resistance management (IRM) is proposed as a strategy to prevent resistance and avoid depleting the susceptibility resource in mosquito populations. IRM strategies assume that alternation of insecticides with different toxicological modes of action will prevent the selection of resistance. Unfortunately, very few field evaluations have reported IRM schemes to control *Ae. aegypti*. In our study, the exclusive use of pyrethroids in vector control programs in Mexico from 1999 to 2013, led in the selection of knockdown resistance (*kdr*) to pyrethroid insecticides. To address this issue, vector control programs temporarily phased out pyrethroids from 2013 to 2019, substituting them with an insecticide class with a different mode of action: organophosphates (OPs). During six years, we monitored pyrethroid resistance in 24 mosquito populations from Tapachula, Mexico. We show that discontinuing pyrethroids for six years led in pyrethroid resistance reversal in *Ae. aegypti* in the field. However, high levels of pyrethroid resistance continue to jeopardize operational application, necessitating longer periods of pyrethroid cessation and novel IRM strategies to achieve lower resistance thresholds.

Introduction

Insecticide spraying is an important tool for mitigating *Aedes* adult populations and reducing the transmission of arboviruses such as dengue, Zika, and chikungunya [1]. The most used adulticides for *Aedes* control programs are classified by their mode of action as sodium channel modulators (e.g., pyrethroids and DDT) or acetylcholinesterase inhibitors (e.g., organophosphates and carbamates) [2, 3]. Because few formulations are approved and available for public health use, the operational tendency in dengue vector control is the use of a chemical class for long periods, resulting in an intense selection for resistance that eventually leads to mosquito control failure. Then, there is a subsequent switch to an alternative insecticide until resistance develops for that insecticide resulting in failure again. To resolve this challenge, insecticide resistance management (IRM) has been proposed as a strategy to retain the use of insecticides on natural populations [4].

IRM involves temporal or spatial rotations among alternative insecticides with different modes of action [3]. Ideally, these schemes reduce selection pressure from any one insecticide for any resistance mechanism [5-7]. Strikingly, few studies have demonstrated the value of IRM schemes in mosquito populations. One of these studies reported that annual rotations and spatial mosaics of pyrethroids (PYR), organophosphates (OP), and carbamates (CARB) reduced PYR resistance in *Anopheles albimanus* in Mexico compared to an exclusive PYR scheme [8, 9]. In *Ae. aegypti*, a 3-month randomized trial in Mexico showed high efficacy of indoor residual spraying of bendiocarb (CARB) compared to deltamethrin (PYR) in a region with widespread PYR resistance [10]. More recently, the impact of varying operational treatment regimens was evaluated in PYR-resistant *Ae. aegypti* populations from Florida over 2.5 years, where PYR susceptibility did not decline [11].

In Mexico, PYR application by dengue control programs for nearly two decades resulted in widespread resistance in *Ae. aegypti* populations [12-14]. From 1999 to 2010, PYR type-1

97 formulations containing permethrin or phenothrin were used intensively. Then, from 2010 to 2013,
98 PYRs type-2, which contain an alpha-cyano moiety, were used briefly. Both PYRs type-1 and
99 type-2 target the voltage-gated sodium channel (VGSC); however, PYR type-2 increased toxicity
100 is due to differences in the way the PYR binds to the VGSC and possibly to additional ion channels
101 [15]. Despite this, two major resistance mechanisms─target site insensitivity and enhanced
102 metabolism—confer resistance to both type-1 and type-2 pyrethroids in *Ae. aegypti* populations
103 in Mexico [12-14]. Target site resistance is associated with amino acid replacements that prevent
104 the PYR from binding to the VGSC, commonly referred to as knockdown resistance mutations
105 (*kdr*) [16-18]. In Mexico, three amino acid replacements— V410L, V1016I, and F1534C—are
106 associated with different levels of pyrethroid resistance [19-22]. The frequency of the three *kdr*
107 mutations increased simultaneously from 2000 to 2013─a period of exclusive PYR use by vector
108 control programs [23]. In addition, enhanced metabolism has been associated with PYR
109 resistance in Mexican *Ae. aegypti* through bioassays and transcription analysis of insecticide
110 detoxification-associated genes such as the carboxyl/cholinesterases (CCE), glutathione-s-
111 transferases (GST), and cytochrome P^{450} monooxygenases (CYP) [24, 25].

112 Mechanisms of resistance, such as *kdr*-conferring mutations in *Ae. aegypti* commonly have
113 been associated with a lower fitness in the absence of insecticides [26-29]. For example, two
114 PYR-resistant laboratory strains from Thailand and Brazil were restored almost to susceptibility
115 after 12 and 15 generations, respectively, without insecticide pressure [26, 27]. Then a semi-field
116 study showed that a PYR-resistant strain from Mexico lost phenotypic resistance in ten
117 generations, whereas *kdr* frequencies were unchanged [28]. Recently, the absence of insecticides
118 for eight generations resulted in a decline in PYR resistance and *kdr*-allele frequencies in *Ae.*
119 *aegypti* from different geographical regions in Mexico [29]. These observations support the
120 potential use of IRM schemes under the assumption that susceptibility alleles will replace
121 resistance-conferring alleles when an insecticide is discontinued.

122 A major goal in this field study is to test whether PYR discontinuation and replacement with
123 alternative chemicals cause the reversal of PYR resistance and *kdr*-conferring mutations in
124 mosquito populations. We conducted a 5-year longitudinal observational study in which the vector
125 control program in Tapachula, Mexico, replaced the use of PYRs with OPs from 2013 to 2019.
126 We surveyed 24 mosquito sites in Tapachula and determined the LC_{50} for permethrin (type-1)
127 and deltamethrin (type-2) and *kdr* allele frequencies every year from 2016 to 2021. Our results
128 show large spatial heterogeneity in PYR resistance across Tapachula. Despite the heavy use of
129 OPs and a lack of control over the domestic use of PYR at our study site, we report a substantial
130 decline in the pyrethroid LC_{50}s and one of the *kdr* alleles over time. Strikingly, the decline in LC_{50}s
131 was rapidly offset by the reintroduction of PYRs in combination with a neonicotinoid. Because few
132 insecticide classes are available for public health use, the evaluation of IRM schemes to maintain
133 insecticide effectiveness is imperative.

134 Methods

135 **Study site.** The city of Tapachula in the state of Chiapas is located in southeastern Mexico near
136 the border with Guatemala. Tapachula is located 177 m above sea level and has a population of
137 305,766 inhabitants within 303 km^2. The city has a tropical climate, with an average maximum
138 temperature of 33 °C, a minimum temperature of 23 °C and an average annual precipitation of
139 1,399.5 mm. Tapachula has two very distinct seasons: the dry season from November to April
140 and the rainy season from May to October.

141 **Insecticide applications by vector control programs.** Tapachula's Vector Control District VII
142 maintains detailed documentation of the insecticide use since 2011. Between 2011 and 2022,
143 insecticide applications were conducted as whole-city or focalized spraying operations. Because
144 Tapachula is a dengue endemic city, whole-city spraying is planned on a yearly basis to reduce
145 mosquito populations from April to November. Every year, four application cycles consisting of
146 four weekly sequential applications cover the city using ultra-low volume spraying (ULV). In

147 contrast, focalized spraying is a reactive strategy to treat households with a “suspected” dengue
148 case. Within a week of the report, dengue “suspected” households are treated with indoor residual
149 spraying and/or intradomicile spatial spraying, and neighboring blocks are treated with ULV. The
150 insecticide applications follow the national guidelines published by Centro Nacional de Prevention
151 y Control de Enfermedades in Mexico (CENAPRECE) [30]. The data was used to summarize the
152 total area treated by each insecticide formulation each year.

153 **Collection sites.** We collected larvae at 24 sites in Tapachula. These sites were located in each
154 of four quadrants: Northwest (NW), Northeast (NE), Southwest (SW), and Southeast (SE). Each
155 collection site consisted of nine consecutive blocks (3 x 3 x 3). One larvitrap (half of a rubber tire)
156 was filled with 3 L of water and suspended from a fence, window, or tree in the front or back yard
157 of individual houses [31]. This collection method proved to be 3.6-fold more efficient than ovitraps
158 to collect *Ae. aegypti* [31]. The water from the larvitraps was poured into plastic containers and
159 transferred to the insectary; the larvitrap was then refilled with 3 L of water. Six houses were
160 included per site, and sites were visited every week for 3 to 4 consecutive weeks or until 500-800
161 *Ae. aegypti* females per site were obtained. Larvae and pupae were transported to the insectary
162 at Centro Regional de Investigación en Salud Pública (CRISP). Adults were identified following
163 emergence, and *Ae. aegypti* were placed in cages (30 cm^3) with other mosquitoes collected from
164 the same site. These adults were allowed to blood feed on a rabbit (under accepted procedures
165 by the Ethical Commission of the Instituto Nacional de Salud Pública), which allowed us to obtain
166 sufficient F_1 and F_2 offspring for the insecticide bioassays. Environmental conditions consisted of
167 27 ± 2 ˚C temperature, 70–80% humidity, and a 12:12 h photoperiod.

168 **Collection times.** At each site, we conducted five collections from 2018 to 2020. Additionally,
169 collections were made at 17 sites in 2016 as a preliminary survey of PYR resistance levels and
170 *kdr* alleles. Because pyrethroids were discontinued by vector control programs in May 2013, we
171 timed each collection according to the number of months after pyrethroid discontinuation. The
172 chronological collections were conducted in September 2016 (38 months after PYR removal),

173 September 2018 (62 months), March 2019 (68 months), September 2019 (74 months), March
174 2020 (80 months), and September 2020 (86 months).

175 **Bioassays.** The F_1 or F_2 adult offspring were exposed to insecticide concentrations using the
176 bottle bioassay procedures described in Solis-Santoyo et al. (2021) [32]. Technical grade
177 insecticides (Supelco) were used to determine the lethal concentration 50 (LC_{50}), which is the
178 amount required to kill 50% of the mosquitoes. We determined the LC_{50} for permethrin (PYR type-
179 1), deltamethrin (PYR type-2), malathion (OP), chlorpyrifos (OP), and bendiocarb (CARB). In this
180 study, we show the results for pyrethroids only. During the bioassay, 15 to 20 females (2–3 days
181 old) were gently aspirated into each bottle. After 1 h of exposure, the mosquitoes were transferred
182 to plastic containers and maintained in the insectary to observe the mortality at 24 h. The LC_{50} of
183 each insecticide was also determined for the NO reference strain every 6 months to confirm
184 insecticide toxicity. The LC_{50}, 95% confidence intervals, slope, intercept, and *p* values were
185 determined using the binary logistic regression model with QCal software [33]. The null hypothesis
186 (Ho) assumed the observed mortality curve adjusts to a binary logistic regression model. Thus,
187 we expected *p* values greater than 0.05 to accept the Ho. When the Ho was rejected, the bioassay
188 was repeated. To estimate the level of resistance among sites, we calculated a resistance ratio
189 (RR) by dividing the LC_{50} of the field sites by the LC_{50} of the NO strain calculated in each period.

190 **Genotyping *kdr* mutations.** Genomic DNA was isolated from 50 individual F_1 female mosquitoes
191 from each collection site following the procedures of Black and DuTeau [34]. The DNA was
192 suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8) and stored at -20 ˚C. The V1016I
193 and F1534C mutations located in the VGSC gene were genotyped according to established
194 protocols [19, 21]. The genotype frequencies at each locus were tested for Hardy-Weinberg (HW)
195 equilibrium. The null hypothesis is that equilibrium is present in the population, which was verified
196 with a chi-square test (df = 1 and *p* value > 0.05). Bayesian 95% Highest Density Intervals (HDI)

197 around frequencies at loci 1016 and 1534 were calculated in WinBUGS for each collection site
198 [35].

199 **Linkage disequilibrium between alleles at loci 1016 and 1534**. Four potential 1016/1534
200 dilocus haplotypes were analyzed: V_{1016}/F_{1534} (VF), V_{1016}/C_{1534} (VC), I_{1016}/F_{1534} (IF), I_{1016}/C_{1534} (IC).
201 The number of times (Tij) that an allele at locus *i* (1016) appears with an allele at locus *j* (1534)
202 and an unbiased estimate of the composite disequilibrium coefficient (Δij) were calculated using
203 the program LINKDIS according the procedures in Vera-Maloof (2015) [17]. A χ^2 test was
204 performed to determine if significant disequilibrium exists among all alleles at loci 1016 and 1534.
205 The statistic was calculated and summed over all two-allele interactions. The linkage
206 disequilibrium correlation coefficient R*ij* is distributed from -1 (both mutations trans) to 0 (the 1534
207 and 1016 alleles occur independently) to 1 (both mutations cis) and therefore provides a
208 standardized measure of disequilibrium.

209 **Generalized logistic regression.** A multivariate logistic regression model tested the relation
210 between the odds of dying (dead/batch) and three independent variables: concentration of
211 insecticide, time (in months), and collection site. The data were obtained from the bottle bioassays
212 in which groups of mosquitoes (batch) were exposed to different concentrations of permethrin
213 and deltamethrin for 1 h, and mortalities were scored at 24 h. The independent variable was the
214 number of dead mosquitoes recorded at 24 h. The experimental unit was a batch of mosquitoes
215 from a "site," collected in a "month," and exposed to a specific concentration of insecticide
216 (µg/bottle). The 24 collection sites across Tapachula were included as nominal variables in the
217 model. Time in "months" was included as numerical variables consisting of the number of months
218 after the discontinuation of pyrethroids by vector control programs. Because, pyrethroid use was
219 reestablished at the end of 2019 (as a mixture of imidacloprid and allethrin), the regression
220 analysis excluded the 80- and 86-month time points. The null hypothesis is that the odds of dying
221 is independent of the insecticide concentration, time, and site. The generalized multinomial linear

222 regression model was conducted using the MASS package in R (glm (dead/batch ~ dose + time
223 + site)), using a binomial distribution.

224 Results

225 Insecticide use by vector control programs in Tapachula

226 From 1999 to 2010, permethrin synergized with piperonyl butoxide (PBO) was used exclusively
227 by vector control programs [13]. Since 2011 CENAPRECE added classes of insecticides with a
228 different mode of action, such as OPs and CARBs [30] to the list of approved insecticides.
229 Tapachula's Vector Control District VII replaced the use of PYR with OPs in May 2013. The
230 number of treated hectares in Tapachula's whole-city and focalized application is shown in
231 Figures 1A and B, respectively. In 2012, whole-city spraying applied phenothrin (PYR) across
232 83,000 hectares. Then, between 2013 and 2022, OPs such as chlorpyrifos-ethyl and malathion
233 were used intensively. In 2019 and 2022, primarily OPs were used; however, applications of OPs
234 were alternated with a mixture of imidacloprid (neonicotinoid) and prallethrin (PYR). In contrast,
235 the number of hectares treated with focalized spraying was three-fold lower than whole-city
236 spraying (Fig 1B). Phenothrin was used until May 2013; in our study, this time is considered month
237 0 after the discontinuation of PYR. From 2013 to 2018, the focalized use of malathion,
238 chlorpyrifos-ethyl, and pirimiphos-methyl was reported. From 2018 to 2022, these OPs were
239 alternated with a mixture of imidacloprid and prallethrin. Between 2020 and 2022, this mixture
240 was used to treat between 25 and 60% of the focalized interventions. Additional insecticides used
241 for indoor residual spraying included propoxur and bendiocarb in 2018; subsequently, bendiocarb
242 was used exclusively in 2019 and 2020.

243 Pyrethroid resistance in *Aedes aegypti* from Tapachula

244 We determined the levels of resistance to permethrin (PYR type-1) and deltamethrin (PYR type-
245 2) in adult females using a customized bottle bioassay. Briefly, we exposed mosquitoes to four to
246 six pyrethroid concentrations for 1 h, then transferred mosquitoes to observation cups and

247 recorded mortality at 24 h. An initial screening in 2016 included ten sites in Tapachula and nine
248 sites across the coast of Chiapas (see coordinates in Table S1). From 2018 to 2021, we
249 conducted recurrent collections at 24 sites in Tapachula (Fig 2). The collections were conducted
250 chronologically in September 2016 (38 months after PYR discontinuation), September 2018 (62
251 months), March 2019 (68 months), September 2019 (74 months), March 2020 (80 months), and
252 September 2020 (86 months). The LC_{50} of each collection site was calculated using a binary
253 logistic regression model and then compared to the New Orleans (NO) susceptible reference LC_{50}
254 to determine the relative resistance ratios (RR).

255 **Permethrin resistance levels.** In our initial screening in 2016, permethrin RRs ranged from 10-
256 to 81-fold. The RRs and their 95% confidence intervals for permethrin and deltamethrin at each
257 collection site and time are shown in Figure 3A. In 2018, the RRs ranged between 7- and 63-fold
258 [32]. In March 2019 and September 2019, the RRs ranged from 7- to 52-fold and from 8- to 42-
259 fold, respectively (Fig 3A). In March 2020, the RRs slightly increased from 22- to 59-fold after
260 PYR was reintroduced by the vector control program in a mixture of imidacloprid and prallethrin
261 (a neonicotinoid and a PYR type-1).

262 **Deltamethrin resistance levels.** In 2016, RRs ranged between 21- and 190-fold (Fig 3B). In
263 2018 and March of 2019, RRs ranged between 11- and 102-fold and 8- and 81-fold, respectively.
264 In September of 2019, we observed the smallest RR range, between 6- and 49-fold, suggesting
265 that the removal of PYR from vector control programs resulted in deltamethrin resistance reversal.
266 However, the RRs observed in March and September of 2020 increased to 22- and 127-fold and
267 22- and 88-fold, respectively. These increases corresponded to the reintroduction of PYR in a
268 mixture of imidacloprid and prallethrin in 2019.

269 **Modeling the odds of dying according to the site, time from PYR-removal, and insecticide**
270 **concentration.** We conducted a multivariate logistic regression to test the relation between the
271 odds of dying and three independent variables: site, time in months, and insecticide concentration.

272 The bioassays provided the number of dead mosquitoes out of the total mosquitoes exposed to
273 a specific concentration of insecticide (dose-response). This strategy allowed us to include the
274 dose-response data from four to six concentrations instead of only using the LC_{50} value, thereby
275 providing more statistical power. Figure 4A shows the proportion of dead individuals by the group
276 of mosquitoes from each collection site and time exposed to the different insecticide
277 concentrations. Note that this figure includes data from cemeteries along the coast of Chiapas
278 (indicated as 'panteon'); however, the generalized linear model included only collection sites
279 within the city of Tapachula from 2016 to March 2020. We excluded coastal sites because
280 insecticide selection pressure is unknown. In addition, we excluded bioassay data from
281 September 2020 and 2021 because, during these years, the pyrethroid prallethrin was
282 reintroduced by the vector control program. The analysis was run separately for permethrin and
283 deltamethrin.

284 For permethrin, 625 dose-response entries were included in the model. Figure 4A shows the
285 proportion of dead mosquitoes for each concentration, site, and time. The multivariate logistic
286 model was significant (*p* value < 2.2e-16), with the odds of dying being explained by the
287 insecticide concentration, the time, and the collection site (Table 1). The McFadden's Pseudo R2
288 for this model was 0.79 (p < 0.000). The estimates for the model (Annex 1) showed that a one-
289 unit increase in one log of permethrin concentration corresponded to an increase in the odds of
290 dying of 814% (*p* value = 2E-16), with site and time remaining constant. One unit of time (one
291 month) corresponded to an increase in the odds of dying by 1.5% (*p* value = 2e-16), with site and
292 concentration remaining constant. Out of 24 site estimates, 18 were significant, with two showing
293 positive and 16 negative directions (Table S2).

294 For deltamethrin, 656 data entries were included in the multivariate regression analysis. The
295 three independent variables (concentration, time, and site) significantly explained the odds of
296 dying (*p* value = 2.2e-16) (Table 1). Figure 4B shows the proportion of dead mosquitoes for each

297 concentration, site, and time. The McFadden's Pseudo R2 for this model was 0.786 ($p < 0.05$).
298 The estimates showed that a one-unit increase in one log of the dose of deltamethrin
299 corresponded to an increase in the odds of dying by 447% (*p* value = 2e-16), with time and site
300 remaining constant. Every month after PYR removal corresponded to an increase in the odds of
301 dying by 8.4% (*p* value = 2e-16), with site and dose remaining constant. Out of 24, 19 site
302 estimates were significant, with six showing positive and 13 negative directions (Table S2).

303 ***Kdr*-allele frequencies over time.** The frequency of two knockdown resistance-associated
304 mutations (*kdr)* in the VGSC were screened in ~50 individuals from each of the 144 collection
305 sites from 2016 to 2020. Genotypic frequencies were calculated for 7,013 individual mosquitoes.
306 At the V1016I locus, alleles segregated in all 144 collection sites. Meanwhile, at the F1534C locus,
307 alleles segregated at 113 sites, mostly because of fixation of the resistant allele at 31 sites.
308 Frequencies of the resistant alleles at loci F1534C (C1534) and V1016I (I1016) and their 95%
309 Bayesian high-density intervals (HDI) are shown in Figure 5. In 2016, we screened ten sites in
310 the city of Tapachula and seven sites along the coast of Chiapas (cemeteries). Nonoverlapping
311 95% HDI suggested significant differences between the city and coastal collections. Resistant
312 I1016 frequencies were significantly higher in the city (mean = 0.48, 95% HDI = 0.45 - 0.507) than
313 along the coast (mean = 0.31, HDI = 0.28 - 0.35). For resistant allele C1534, city frequencies
314 were also higher (mean = 0.97, 95% HDI = 0.968 - 0.983) than along the coast (mean = 0.889,
315 95%HDI = 0.86 - 0.91). Because of these differences between city and coastal sites, the
316 multivariate regression analysis included only the city collection sites from 2016 to 2020. For
317 easier visualization, the resistant allele frequency was summarized by quadrant at the different
318 collection times (Fig S1). The C1534 allele frequency was close to fixation in 2016, 2018, 2019-
319 M, and 2019-S (mean allele frequency from 0.93 to 0.98). Then, from March to September of
320 2020, the mean frequency declined from 0.91 to 0.84 (95%HDI = 0.83 - 0.86), respectively. For
321 I1016, no clear tendency for decline was observed from 2016 to 2019; however, in March 2020,

322 the frequencies were significantly higher than at other collection times. These higher frequencies
323 seemed to be a response to the reintroduction of pyrethroids in vector control programs.

324 **Relation between LC_{50} and *kdr*-alleles.** In Mexican mosquito populations, C1534 and I1016
325 occurred together more often than expected [17]. In our linkage disequilibrium analysis among
326 144 collections, resistant alleles at C1543 and I1016 were not in disequilibrium, possibly because
327 C1534 was close to fixation in most of the collections. To test whether a particular dilocus
328 genotype explains the variation in LC_{50}s, we conducted a multivariate regression analysis using
329 LC_{50} as the response. Independent variables were site, month, and the proportion of each of the
330 nine possible dilocus genotype combinations (Fig 6). The first two letters correspond to the 1016
331 locus and the second two letters to locus 1534. An individual with homozygote-susceptible
332 genotypes at both loci (V1016I and F1534C) were designated as VVFF, whereas the double-
333 resistant homozygote was assigned IICC. The most common dilocus genotypes among
334 collections were IVCC, IICC, and VVCC (Fig 6). From 2016 to 2018, the frequency of the resistant
335 IICC genotype declined, whereas VVCC increased. From March 2019 to September 2020, IICC
336 increased again. The increase seemed to be a response to pyrethroid reintroduction in vector
337 control programs in 2019 and 2020. Then, in September 2020, IICC decreased significantly. The
338 model for permethrin included 74 sites with phenotypes and genotypes. The permethrin LC_{50}s
339 were significantly explained by the VVCC haplotype (estimate = -1.465, T = -2.192, *p* value =
340 0.0337). For deltamethrin, LC_{50}s were only significantly explained by the month (estimate = -
341 0.056, T = -7.42, *p* value = 2.7E-09).

342 Discussion

343 We tested the hypothesis that PYR resistance carries negative fitness and that susceptibility is
344 restored in field mosquito populations when pyrethroids are not used anymore. To account for the
345 large heterogeneity in field mosquito populations, we screened 24 collection sites every year in
346 Tapachula. Our results indicate that mosquitoes from the 24 sites became more susceptible to

347 pyrethroids over time. The model detected that with every passing month after the discontinuation
348 of pyrethroids by vector control programs, mortality increased by 8.4% and 1.5% for deltamethrin
349 and permethrin, respectively. This decline in resistance was also evident for one of the *kdr*
350 mutations, for which the resistant C1534 allele declined 12% after near fixation in the initial part
351 of our study. These results were expected, as several laboratory investigations have
352 demonstrated that PYR resistance reversed in less than 15 generations in the absence of
353 insecticides. However, few studies have evaluated the response of mosquito populations in the
354 field where mosquitoes are under different insecticide selection pressures. Recently, one study in
355 Florida measured the phenotypic and genotypic response of PYR-resistant *Ae. aegypti*
356 populations following 2.5 years of the operational use of organophosphates [11]. The results did
357 not detect a decline in diagnostic times with the CDC bottle bioassay; however, the authors
358 observed a significant decline in the frequency of double homozygous *kdr* resistant genotypes
359 (V1016I and F1534C). In our study, however, we identified decreases in both the pyrethroid LC_{50}s
360 and *kdr* genotypes. One potential explanation for these differences is that the methods used to
361 measure resistance targeted different resistant phenotypes. The study in Florida used a time-
362 response assay to measure knockdown in mosquitoes at 30 min of exposure. In comparison, the
363 dose-response assay in our study measured the mortality at 24 h using different pyrethroid
364 concentrations. Although, the CDC bottle bioassay has shown greater variation in the dose–
365 response curves in comparison with the WHO tube test and the topical application bioassay [36],
366 scoring mortality at 24 h instead of 30 min is a better predictor of resistance because it accounts
367 for recovery due to metabolic resistance mechanisms [37]. Whether the dose-response assays
368 are more sensitive than the time-response assays to identify changes in pyrethroid resistance is
369 of special interest, as the interpretation of results might have important implications at the
370 operational level.

371 Despite the significant decline in pyrethroid LC_{50}s over time, our results show that the RR levels
372 are still higher than 10-fold almost 8 years after the discontinuation of PYR. This finding is
373 consistent with an observational study in Sao Paulo, Brazil, in which levels of PYR resistance
374 remained high 10 years after the switch to OPs to control *Ae. aegypti* [38]. The authors suggested
375 that pyrethroid resistance remained high in mosquito populations because of the widespread use
376 of pyrethroids in consumer-based products for domestic and residential application. For instance,
377 a survey in the city of Merida, Mexico, showed that 87% of households regularly use pyrethroid-
378 based commercial products to reduce mosquito nuisance [39]. Moreover, the use of PYR surface
379 sprays led to a significant increase in the frequency of V1016I *kdr* homozygotes in surviving *Ae.*
380 *aegypti*, suggesting strong selection pressure for this allele [40]. Strikingly, even when our study
381 did not account for the use of pyrethroid-based commercial products and despite the large
382 heterogeneity in PYR resistance across the 24 sites, our model was able to identify a decline in
383 PYR resistance over time. A limitation in our model is that we cannot predict the time required to
384 reach lower levels of resistance (< 5-fold), which would require continuing to monitor for resistance
385 for several years. Another limitation is that our surveillance began 3 years after the discontinuation
386 of the PYRs, and we lack information about the PYR resistance levels during the peak of PYR
387 use in Tapachula. Our closest references are three studies that evaluated *Ae. aegypti* PYR
388 resistance in Mexico using the same methods. In 2007, five collection sites in the states of
389 Quintana Roo and Yucatan showed permethrin RRs ranging between 2.6- and 10.2-fold [24]. In
390 2009, seven sites in the state of Veracruz showed deltamethrin RRs were lower than 3-fold at
391 four sites and between 16- and 20-fold at three sites. In the same study, permethrin RRs were
392 lower than 10-fold at four sites and between 11- and 33-fold at three sites [13]. In 2014, eight sites
393 from the states of Yucatan, Guerrero, and Chiapas showed deltamethrin RRs ranging between
394 18- and 108-fold whereas permethrin RRs ranged between 15- and 60-fold. In this last study,
395 Tapachula showed RRs of 25- and 95-fold for permethrin and deltamethrin, respectively [29].
396 These findings suggest high levels of pyrethroid RRs were widespread just before our study.

397 However, because large heterogeneity in pyrethroid RRs can be found at the city scale [32],
398 making assumptions about resistance levels across states and different studies should be done
399 with caution.

400 Interestingly, our RRs were higher for deltamethrin (PYR type-2) than for permethrin (PYR
401 type-1). This finding was unexpected, as mosquito populations were subject to PYR type-1 rather
402 than PYR type-2 from 1999 to 2013. One possible explanation could be that mosquito populations
403 were exposed to PYR type-2 selection from the use of consumer-based pyrethroid products.
404 Future studies should evaluate the use of these products at the study sites. A second observation
405 was that PYR discontinuation by vector control programs resulted in higher deltamethrin decline
406 rates than those rates observed for permethrin; however, *kdr* dilocus genotypes did not explain
407 the variation. These results suggest a possible higher fitness cost for deltamethrin resistance in
408 the absence of insecticides. Specific mechanisms of resistance for pyrethroids type-1 and type-2
409 might include a combination of several mechanisms such as specific *kdr* mutations, compensatory
410 ion channel mutations, cuticle modification, and specific enzyme detoxifying variants with different
411 fitness costs in mosquitoes. A recent study showed that metabolic resistance was associated with
412 higher fitness costs than target-site resistance in the absence of insecticides [41]. From the three
413 congenic *Ae. aegypti* strains carrying either CYP-mediated, *kdr*-mediated, or CYP + *kdr*
414 mechanisms, strains with CYP-mediated resistance showed a significantly reduced net
415 reproductive rate relative to the susceptible and the *kdr* strains [41]. In our study, we observed a
416 major decline in PYR RRs from 2016 to 2019, which did not match the decline in *kdr* alleles that
417 occurred mostly from 2019 to 2020. We hypothesize that metabolic resistance mechanisms or
418 epistatic factors might have had a greater impact on the resistance levels from 2016 to 2019. A
419 side project measured the enzymatic activity of CCE, CYP, and GST in collections taken in 2018
420 and 2020 from 22 sites in Tapachula [42]. The number of sites with mean enzymatic activity higher
421 than the susceptible New Orleans (NO) reference strain declined for alpha-, beta-, and PNPA-

422 esterases at three, one, and five collection sites, respectively. In addition, an increase was
423 observed in the number of sites with higher GST and CYP activity than the susceptible strain. The
424 enzyme activity levels were not significantly associated with PYR RRs, but bioassays using PYRs
425 in combination with enzyme inhibitors significantly increased the mortality, suggesting
426 biochemical assays might not be sensitive enough to explain PYR resistance. Including DNA or
427 RNA markers of insecticide detoxification genes might be a good option to explore the role of
428 metabolic resistance in mosquito populations. However, this requires a fine understanding of the
429 genes conferring resistance to a particular insecticide in a geographical region and their variation
430 within and between populations [24]. For instance, single nucleotide polymorphisms (SNPs) in
431 detoxification genes have been associated with PYR resistance in our study sites, and we are in
432 the process of testing their segregation and association with different levels of resistance in field
433 mosquitoes [43].

434 Many studies have intended to use *kdr* frequencies as a proxy for pyrethroid resistance.
435 Particularly in this study, *kdr* mutations explained somewhat the large variation among pyrethroid
436 RRs. Only one dilocus genotype (VVCC) significantly explained the large variation in permethrin
437 LC_{50}s; however, no *kdr* dilocus genotype explained deltamethrin LC_{50}s. From 2016 to 2019,
438 C1534 allele frequencies had low spatial and temporal heterogeneity because of proximity to
439 fixation, whereas I1016 frequencies had large spatial heterogeneity within a 0.35 to 0.68 range.
440 The lack of association between the PYR LC_{50}s and *kdr*-dilocus genotypes could be because the
441 method to measure resistance in our study (24-h mortality) was not only measuring *kdr* but also
442 was measuring recovery mechanisms due to metabolic resistance. Recovery rates among
443 knocked-down mosquitoes ranged between 30 and 45% at our study sites. A previous genome-
444 wide association mapping from pools of mosquitoes exposed to a permethrin LC_{50} showed that
445 mosquitoes exhibiting knockdown resistance at 1 h of exposure were strongly associated with *kdr*
446 mutations (V410L and V1016I), whereas mosquitos recovering at 4 h were associated with SNPs

447 at a different group of ion channels and detoxifying enzyme-coding genes [44]. The operational
448 implication of mosquito recovery has not been evaluated in the field, and its role in selection of
449 pyrethroid resistance mechanisms might be underestimated.

450 One important observation in our study is that significant changes in *kdr* frequencies occurred
451 between 2020 and 2021, with C1534 decreasing by 6%, whereas I1016 increased by 15%. The
452 increase in I1016 frequencies occurred simultaneously with an increase in pyrethroid LC_{50}s,
453 following the use of imidacloprid and prallethrin (PYR type-1) during whole-city spraying and focal
454 indoor spatial spraying from 2019 to 2020. Such a rapid increase was also documented in Iquitos,
455 Peru, where *kdr* frequencies and heterogeneity increased following a 6-week cypermethrin spray
456 application in 2013 and 2014 [45]. The rapid response in resistance indicated a strong impact of
457 vector control activities on the susceptibility of mosquito populations. Similarly, our study
458 illustrates how short periods of pyrethroid use offset the trends in PYR resistance reversal.
459 Inevitably, IRM strategies will require long-term commitments between vector control programs
460 and community participation to manage the use of pyrethroids and allow the natural course of
461 pyrethroid reversal in resistant populations over time.

462 Conclusion

463 The trends in insecticide resistance in our study followed the application of insecticides by vector
464 control programs, suggesting that operational control seems to have a significant impact in
465 resistance levels across the mosquito collections. Our results in the field support the hypothesis
466 that PYR resistance has negative fitness in the absence of pyrethroids. We observed a decline in
467 PYR resistance over time despite high heterogeneity, heavy application of OPs, and possibly
468 residential-level use of consumer-based PYR products. Despite the significant decline in the
469 range of PYR RRs, resistance is still considered high. Whether the PYR RRs calculated in the
470 laboratory are associated with differences in operational effectiveness requires further evaluation.

Our results suggest that pyrethroid reversal is possible; however, long-term IRM by vector control programs and community participation are needed to speed the process.

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Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of CDC.

481 ## Figure legends

482 **Fig 1. Number of treated hectares during insecticide interventions in Tapachula from 2011**
483 **to 2022:** A) Whole-city spraying and, B) Focalized spraying. Data provided by Vector Control
484 District VII in Tapachula, Chiapas.

485 **Fig 2. *Aedes aegypti* collection sites in Tapachula, Chiapas, from 2018 to 2021**. Larvitraps
486 were used to collect 500-800 females per site to generate the F_1 offspring for insecticide
487 bioassays and kdr genotyping.

488 **Fig 3. Pyrethroid resistance ratios in *Aedes aegypti* collection sites from 2016 to 2021:** A)
489 Permethrin and, B) Deltamethrin. Bars represent the 95% confidence intervals. Sites are
490 categorized within four geographical quadrants in Tapachula (NW = northwest, NE = northeast,
491 SW = southwest, and SE = southeast). Collections from across coastal Chiapas were obtained
492 from cemeteries (panteon). M = March and S = September. Resistance ratios are relative to the
493 New Orleans LC_{50} calculated at each time point.

494 **Fig 4. Proportion of dead mosquitoes from a specific site, collection time, and**
495 **concentration for:** A) Permethrin and, B) Deltamethrin. Orange-scale colors represent the
496 number of months after PYRs were discontinued by vector control programs.

497 **Fig 5. Allele frequency of *kdr* mutations V1016I and F1534C in *Aedes aegypti* from**
498 **Tapachula and coastal Chiapas over time**. Approximately 50 mosquitoes were genotyped to
499 calculate the allele frequencies at each site and time point. Sites are categorized in four
500 geographical quadrants in Tapachula (NW = northwest, NE = northeast, SW = southwest, and SE
501 = southeast). Collections across coastal Chiapas were obtained from cemeteries (panteon). M =
502 March and S = September. Bars represent the 95% HDI around the frequency.

503

504 **Fig 6. Proportion of mosquitoes carrying each of the nine dilocus genotypes over 5 years**
505 **of study.** The number of mosquitoes analyzed per year ranged from 714 to 1200. The first two
506 letters correspond to the amino acid at the V1016I locus (resistant = I, susceptible = V) and the
507 second pair corresponds to the amino acid at F1534C locus (resistant = C, susceptible = F).
508 Dilocus genotypes go from double-resistant homozygote = IICC, to double-susceptible
509 homozygote = VVFF.

510

511 ## Supplementary Files

512 **Supplementary Fig 1S. Allele frequency of *kdr*-mutations C1534 and I1016 in *Aedes***
513 ***aegypti* from Tapachula over time**. Frequency and 95% HDI were calculated yearly from
514 samples that ranged from 714 to 1200 mosquitoes (~50 mosquitoes per collection site). Sites
515 are categorized in four geographical quadrants in Tapachula (NW = northwest, NE = northeast,
516 SW = southwest, and SE = southeast). Circles correspond to C1534 allele frequency and
517 triangles to I1016 allele frequency.

518

519 **Supplementary Table S1. Location of *Aedes aegypti* cemetery collections in the coast of**
520 **Chiapas.** Larvae were collected from each cemetery on 2016's wet season. The municipality
521 name and geographical coordinates are provided.

522

523 **Supplementary Table S2. Summary of generalized linear model estimates for A)**
524 **Permethrin and, B) Deltamethrin.** Independent variables include the insecticide concentration
525 (log(concentration)), site (24 sites), time in months (after PYR discontinuation). Estimates,
526 standard error, z-value, probability and change are shown for each variable.

527 ## References

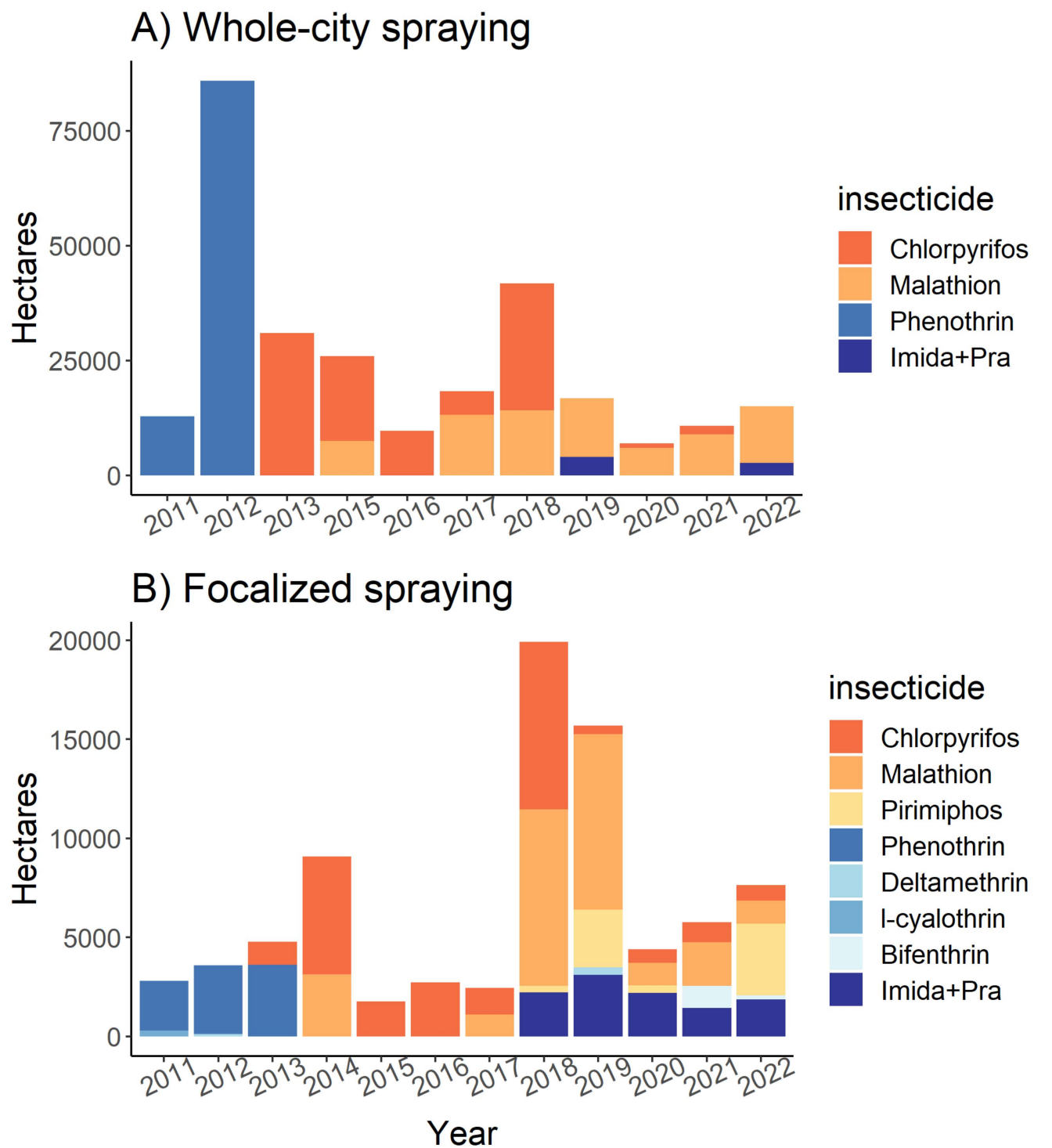
528 1. Moyes CL, Vontas J, Martins AJ, Ng LC, Koou SY, Dusfour I, et al. Contemporary status of
529 insecticide resistance in the major Aedes vectors of arboviruses infecting humans. PLoS Negl Trop Dis.
530 2017;11(7):e0005625. Epub 2017/07/21. doi: 10.1371/journal.pntd.0005625. PubMed PMID: 28727779;
531 PubMed Central PMCID: PMCPMC5518996.
532 2. van den Berg H, da Silva Bezerra HS, Al-Eryani S, Chanda E, Nagpal BN, Knox TB, et al. Recent
533 trends in global insecticide use for disease vector control and potential implications for resistance
534 management. Sci Rep. 2021;11(1):23867. Epub 2021/12/15. doi: 10.1038/s41598-021-03367-9. PubMed
535 PMID: 34903838; PubMed Central PMCID: PMCPMC8669011.
536 3. McCaffery A, R N. The Insecticide Resistance Action Committee (IRAC): Public responsibility and
537 enlightened industrial self interest. Outlooks on Pest Management. 2006;2:11-4.
538 4. Dusfour I, Vontas J, David JP, Weetman D, Fonseca DM, Corbel V, et al. Management of
539 insecticide resistance in the major Aedes vectors of arboviruses: Advances and challenges. PLoS Negl
540 Trop Dis. 2019;13(10):e0007615. Epub 2019/10/11. doi: 10.1371/journal.pntd.0007615. PubMed PMID:
541 31600206; PubMed Central PMCID: PMCPMC6786541.
542 5. Curtis CF. Possible Methods of Inhibiting or Reversing the Evolution of Insecticide Resistance in
543 Mosquitos. Pestic Sci. 1981;12(5):557-64. doi: DOI 10.1002/ps.2780120513. PubMed PMID:
544 WOS:A1981MN84800012.
545 6. Curtis CF. Theoretical-Models of the Use of Insecticide Mixtures for the Management of
546 Resistance. B Entomol Res. 1985;75(2):259-65. doi: Doi 10.1017/S0007485300014346. PubMed PMID:
547 WOS:A1985AME7500008.
548 7. Brogdon WG, McAllister JC. Insecticide resistance and vector control. Emerg Infect Dis.
549 1998;4(4):605-13. doi: DOI 10.3201/eid0404.980410. PubMed PMID: WOS:000077532500010.
550 8. Hemingway J, Penilla RP, Rodriguez AD, James BM, Edge W, Rogers H, et al. Resistance
551 management strategies in malaria vector mosquito control. A large-scale field trial in Southern Mexico.
552 Pestic Sci. 1997;51(3):375-82. doi: Doi 10.1002/(Sici)1096-9063(199711)51:3<375::Aid-Ps636>3.0.Co;2-
553 K. PubMed PMID: WOS:A1997YJ39800025.
554 9. Penilla RP, Rodriguez AD, Hemingway J, Torres JL, Arredondo-Jimenez JI, Rodriguez MH.
555 Resistance management strategies in malaria vector mosquito control. Baseline data for a large-scale
556 field trial against Anopheles albimanus in Mexico. Medical and Veterinary Entomology. 1998;12(3):217-
557 33. doi: DOI 10.1046/j.1365-2915.1998.00123.x. PubMed PMID: WOS:000075782800001.
558 10. Vazquez-Prokopec GM, Medina-Barreiro A, Che-Mendoza A, Dzul-Manzanilla F, Correa-Morales
559 F, Guillermo-May G, et al. Deltamethrin resistance in Aedes aegypti results in treatment failure in
560 Merida, Mexico. PLoS Negl Trop Dis. 2017;11(6):e0005656. Epub 2017/06/13. doi:
561 10.1371/journal.pntd.0005656. PubMed PMID: 28604781; PubMed Central PMCID: PMCPMC5481028.
562 11. Casey Parker-Crockett, Aaron Lloyd, Daviela Ramirez, Connelly CR. Impacts of differential
563 mosquito control treatment regimens on insecticide susceptibility status of Aedes aegypti (Diptera:
564 Culicidae). SN Applied Sciences. 2022;4(249). doi: https://doi.org/10.1007/s42452-022-05130-9.
565 12. Aponte A, Penilla P, F. D-M, A. C-M, D. LA, F. S, et al. The pyrethroid resistance status and
566 mechanisms in *Aedes aegypti* from the Guerrero state, Mexico. Pesticide Biochemistry and Physiology
567 2013;107:226-34.
568 13. Flores AE, Ponce G, Silva BG, Gutierrez SM, Bobadilla C, Lopez B, et al. Wide spread cross
569 resistance to pyrethroids in Aedes aegypti (Diptera: Culicidae) from Veracruz state Mexico. J Econ
570 Entomol. 2013;106(2):959-69. Epub 2013/06/22. PubMed PMID: 23786088; PubMed Central PMCID:
571 PMCPMC3980443.
572 14. Kuri-Morales PA, Correa-Morales F, Gonzalez-Acosta C, Moreno-Garcia M, Santos-Luna R,
573 Roman-Perez S, et al. Insecticide susceptibility status in Mexican populations of Stegomyia aegypti (=

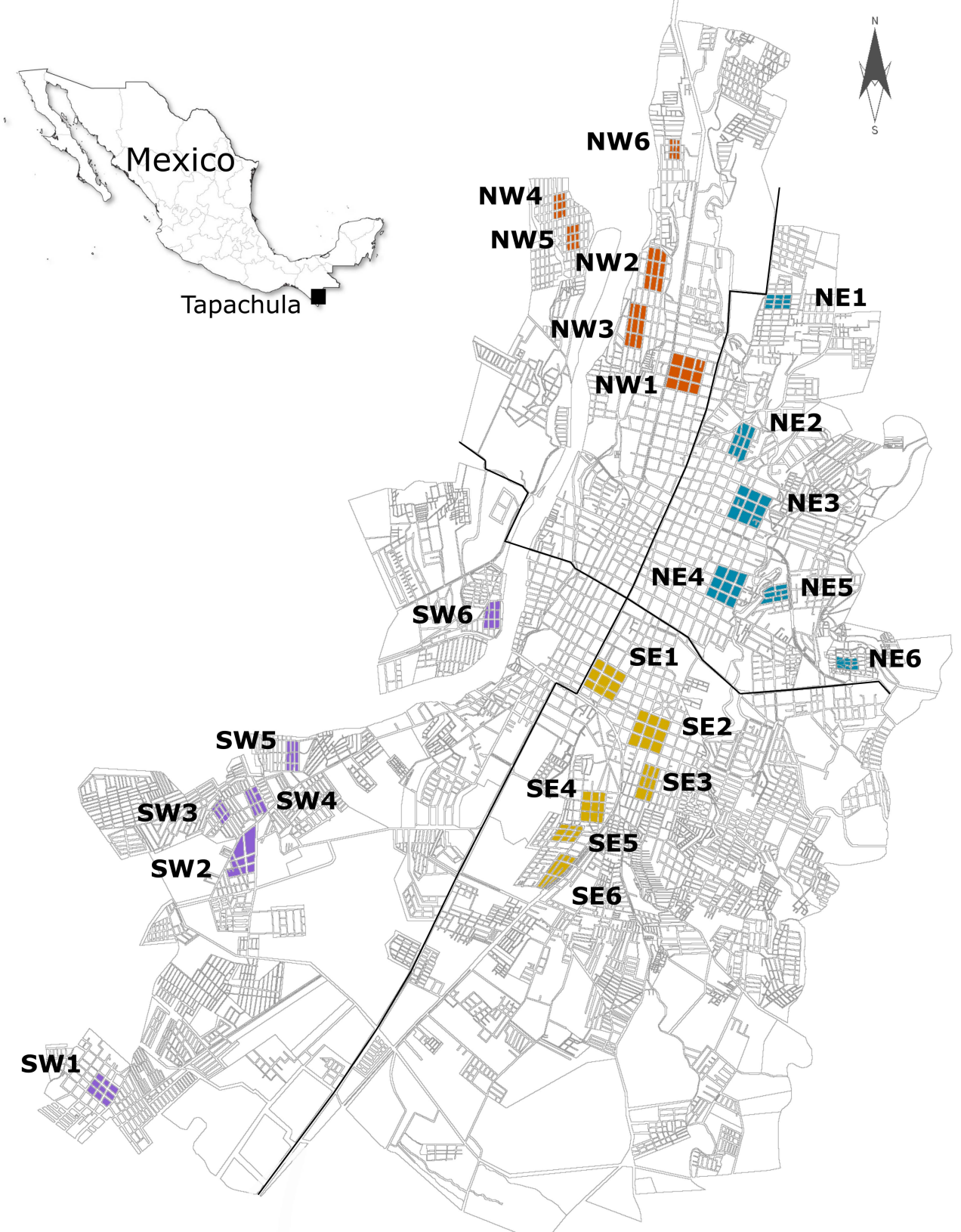
574 Aedes aegypti): a nationwide assessment. Med Vet Entomol. 2018;32(2):162-74. Epub 2017/11/23. doi:
575 10.1111/mve.12281. PubMed PMID: 29165810.
576 15. Soderlund DM, Knipple DC. The molecular biology of knockdown resistance to pyrethroid
577 insecticides. Insect Biochem Mol Biol. 2003;33(6):563-77. Epub 2003/05/29. PubMed PMID: 12770575.
578 16. Garcia GP, Flores AE, Fernandez-Salas I, Saavedra-Rodriguez K, Reyes-Solis G, Lozano-Fuentes S,
579 et al. Recent rapid rise of a permethrin knock down resistance allele in Aedes aegypti in Mexico. PLoS
580 Negl Trop Dis. 2009;3(10):e531. Epub 2009/10/16. doi: 10.1371/journal.pntd.0000531. PubMed PMID:
581 19829709; PubMed Central PMCID: PMCPMC2759509.
582 17. Vera-Maloof FZ, Saavedra-Rodriguez K, Elizondo-Quiroga AE, Lozano-Fuentes S, Black Iv WC.
583 Coevolution of the Ile1,016 and Cys1,534 Mutations in the Voltage Gated Sodium Channel Gene of
584 Aedes aegypti in Mexico. PLoS Negl Trop Dis. 2015;9(12):e0004263. Epub 2015/12/15. doi:
585 10.1371/journal.pntd.0004263. PubMed PMID: 26658798; PubMed Central PMCID: PMCPMC4684211.
586 18. Saavedra-Rodriguez K, Maloof FV, Campbell CL, Garcia-Rejon J, Lenhart A, Penilla P, et al.
587 Parallel evolution of vgsc mutations at domains IS6, IIS6 and IIIS6 in pyrethroid resistant Aedes aegypti
588 from Mexico. Scientific Reports. 2018;8(1):6747. doi: 10.1038/s41598-018-25222-0.
589 19. Saavedra-Rodriguez K, Urdaneta-Marquez L, Rajatileka S, Moulton M, Flores AE, Fernandez-Salas
590 I, et al. A mutation in the voltage-gated sodium channel gene associated with pyrethroid resistance in
591 Latin American Aedes aegypti. Insect Mol Biol. 2007;16(6):785-98. Epub 2007/12/21. doi:
592 10.1111/j.1365-2583.2007.00774.x. PubMed PMID: 18093007.
593 20. Haddi K, Tome HVV, Du Y, Valbon WR, Nomura Y, Martins GF, et al. Detection of a new
594 pyrethroid resistance mutation (V410L) in the sodium channel of Aedes aegypti: a potential challenge
595 for mosquito control. Sci Rep. 2017;7:46549. Epub 2017/04/20. doi: 10.1038/srep46549. PubMed PMID:
596 28422157; PubMed Central PMCID: PMCPMC5396194.
597 21. Yanola J, Somboon P, Walton C, Nachaiwieng W, Somwang P, Prapanthadara LA. High-
598 throughput assays for detection of the F1534C mutation in the voltage-gated sodium channel gene in
599 permethrin-resistant Aedes aegypti and the distribution of this mutation throughout Thailand. Trop Med
600 Int Health. 2011;16(4):501-9. Epub 2011/02/24. doi: 10.1111/j.1365-3156.2011.02725.x. PubMed PMID:
601 21342372.
602 22. Du Y, Nomura Y, Satar G, Hu Z, Nauen R, He SY, et al. Molecular evidence for dual pyrethroid-
603 receptor sites on a mosquito sodium channel. Proc Natl Acad Sci U S A. 2013;110(29):11785-90. Epub
604 2013/07/04. doi: 10.1073/pnas.1305118110. PubMed PMID: 23821746; PubMed Central PMCID:
605 PMCPMC3718148.
606 23. Saavedra-Rodriguez K, Maloof FV, Campbell CL, Garcia-Rejon J, Lenhart A, Penilla P, et al.
607 Parallel evolution of vgsc mutations at domains IS6, IIS6 and IIIS6 in pyrethroid resistant Aedes aegypti
608 from Mexico. Sci Rep. 2018;8(1):6747. Epub 2018/05/02. doi: 10.1038/s41598-018-25222-0. PubMed
609 PMID: 29712956; PubMed Central PMCID: PMCPMC5928250.
610 24. Saavedra-Rodriguez K, Suarez AF, Salas IF, Strode C, Ranson H, Hemingway J, et al. Transcription
611 of detoxification genes after permethrin selection in the mosquito Aedes aegypti. Insect Mol Biol.
612 2012;21(1):61-77. Epub 2011/10/29. doi: 10.1111/j.1365-2583.2011.01113.x. PubMed PMID: 22032702;
613 PubMed Central PMCID: PMCPMC3540788.
614 25. Strode C, Wondji CS, David JP, Hawkes NJ, Lumjuan N, Nelson DR, et al. Genomic analysis of
615 detoxification genes in the mosquito Aedes aegypti. Insect Biochem Mol Biol. 2008;38(1):113-23. Epub
616 2007/12/12. doi: 10.1016/j.ibmb.2007.09.007. PubMed PMID: 18070670.
617 26. Brito LP, Linss JG, Lima-Camara TN, Belinato TA, Peixoto AA, Lima JB, et al. Assessing the effects
618 of Aedes aegypti kdr mutations on pyrethroid resistance and its fitness cost. PLoS One.
619 2013;8(4):e60878. Epub 2013/04/18. doi: 10.1371/journal.pone.0060878. PubMed PMID: 23593337;
620 PubMed Central PMCID: PMCPMC3620451.

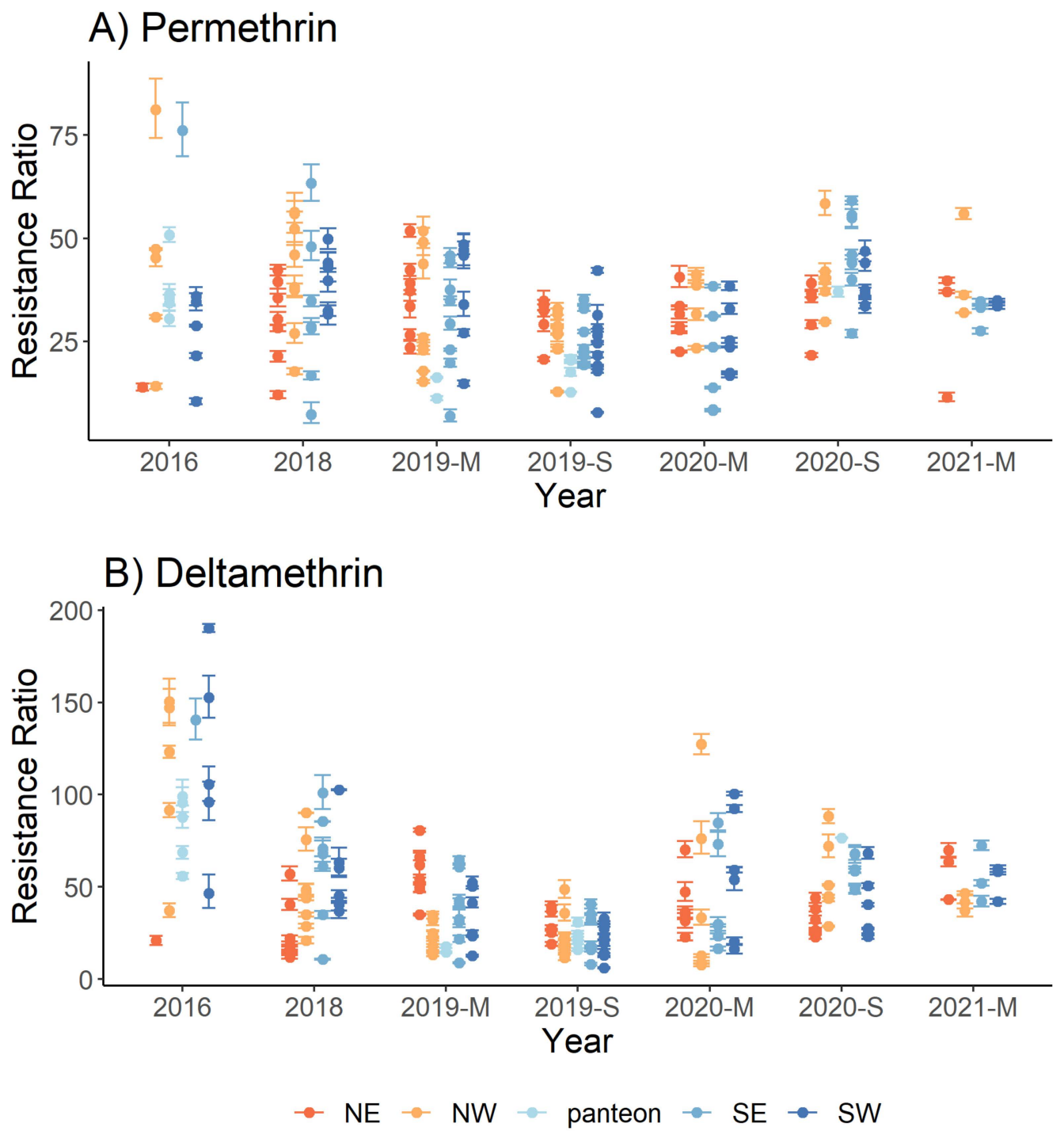
621 27. Cheng Chang, Xin-Yi Huang, Pin-Chun Chang, Huai-Hui Wu, Shu-MeiDai. Inheritance and stability
622 of sodium channel mutations associated with permethrin knockdown resistance in Aedes aegypti.
623 Pesticide Biochemistry and Physiology. 2012;104(2). doi: https://doi.org/10.1016/j.pestbp.2012.06.003.
624 28. Grossman MK, Uc-Puc V, Rodriguez J, Cutler DJ, Morran LT, Manrique-Saide P, et al. Restoration
625 of pyrethroid susceptibility in a highly resistant Aedes aegypti population. Biol Lett. 2018;14(6). Epub
626 2018/06/15. doi: 10.1098/rsbl.2018.0022. PubMed PMID: 29899128; PubMed Central PMCID:
627 PMCPMC6030600.
628 29. Vera-Maloof FZ, Saavedra-Rodriguez K, Penilla-Navarro RP, A DR-R, Dzul F, Manrique-Saide P, et
629 al. Loss of pyrethroid resistance in newly established laboratory colonies of Aedes aegypti. PLoS Negl
630 Trop Dis. 2020;14(3):e0007753. Epub 2020/03/17. doi: 10.1371/journal.pntd.0007753. PubMed PMID:
631 32176693; PubMed Central PMCID: PMCPMC7117762.
632 30. Federacion DDOdl. NOM-EM-003-SSA2–2010 para la vigilancia epidemiologica. Prevencion y
633 control de enfermedades transmitidas por vector. [NOM-032-SSA-2–2002 for epidemiological
634 monitoring. Prevention and control of vector-transmitted diseases]. DOF, Mexico. 2011.
635 31. Quezada-Yaguachi WE, Rodriguez AD, Solís-Santoyo F, Lopez-Solis AD, IV WCB, Saavedra-
636 Rodriguez K, et al. Comparative Evaluation of the Regular Ovitrap vs an Innovated Larvitrap for Aedes
637 Entomological Surveillance in Tapachula. Advances in Entomology. 2022;10:77-84. doi: DOI:
638 10.4236/ae.2022.101006 J.
639 32. Solis-Santoyo F, Rodriguez AD, Penilla-Navarro RP, Sanchez D, Castillo-Vera A, Lopez-Solis AD, et
640 al. Insecticide resistance in Aedes aegypti from Tapachula, Mexico: Spatial variation and response to
641 historical insecticide use. PLoS Negl Trop Dis. 2021;15(9):e0009746. Epub 2021/09/28. doi:
642 10.1371/journal.pntd.0009746. PubMed PMID: 34570792; PubMed Central PMCID: PMCPMC8475978
643 Rodriguez was unable to confirm their authorship contributions. On their behalf, the corresponding
644 author has reported their contributions to the best of their knowledge.
645 33. Lozano-Fuentes S, Saavedra-Rodriguez K, Black WCt, Eisen L. QCal: a software application for the
646 calculation of dose-response curves in insecticide resistance bioassays. J Am Mosq Control Assoc.
647 2012;28(1):59-61. Epub 2012/04/27. doi: 10.2987/11-6192.1. PubMed PMID: 22533088.
648 34. Black IV WC, DuTeau NM. RAPD-PCR and SSCP analysis for insect population genetic studies. The
649 Molecular Biology of Insect Disease Vectors: A Methods Manual. Crampton J BC LC, editor, editor. New
650 York: Chapman and Hall; 1997.
651 35. Lunn, A T, N B, D S. WinBUGS—A Bayesian modelling framework: Concepts, structure, and
652 extensibility. Statistics and Computing. 2000;10(4):325-37.
653 36. Althoff RA, Huijben S. Comparison of the variability in mortality data generated by CDC bottle
654 bioassay, WHO tube test, and topical application bioassay using Aedes aegypti mosquitoes. Parasit
655 Vectors. 2022;15(1):476. Epub 2022/12/21. doi: 10.1186/s13071-022-05583-2. PubMed PMID:
656 36539831; PubMed Central PMCID: PMCPMC9769033.
657 37. Owusu HF, Jancaryova D, Malone D, Muller P. Comparability between insecticide resistance
658 bioassays for mosquito vectors: time to review current methodology? Parasit Vectors. 2015;8:357. Epub
659 2015/07/08. doi: 10.1186/s13071-015-0971-6. PubMed PMID: 26148484; PubMed Central PMCID:
660 PMCPMC4492098.
661 38. Macoris ML, Martins AJ, Andrighetti MTM, Lima JBP, Valle D. Pyrethroid resistance persists after
662 ten years without usage against Aedes aegypti in governmental campaigns: Lessons from Sao Paulo
663 State, Brazil. PLoS Negl Trop Dis. 2018;12(3):e0006390. Epub 2018/03/31. doi:
664 10.1371/journal.pntd.0006390. PubMed PMID: 29601580; PubMed Central PMCID: PMCPMC5895049.
665 39. Lorono-Pino MA, Chan-Dzul YN, Zapata-Gil R, Carrillo-Solis C, Uitz-Mena A, Garcia-Rejon JE, et al.
666 Household use of insecticide consumer products in a dengue-endemic area in Mexico. Trop Med Int
667 Health. 2014;19(10):1267-75. Epub 2014/07/22. doi: 10.1111/tmi.12364. PubMed PMID: 25040259;
668 PubMed Central PMCID: PMCPMC4167961.

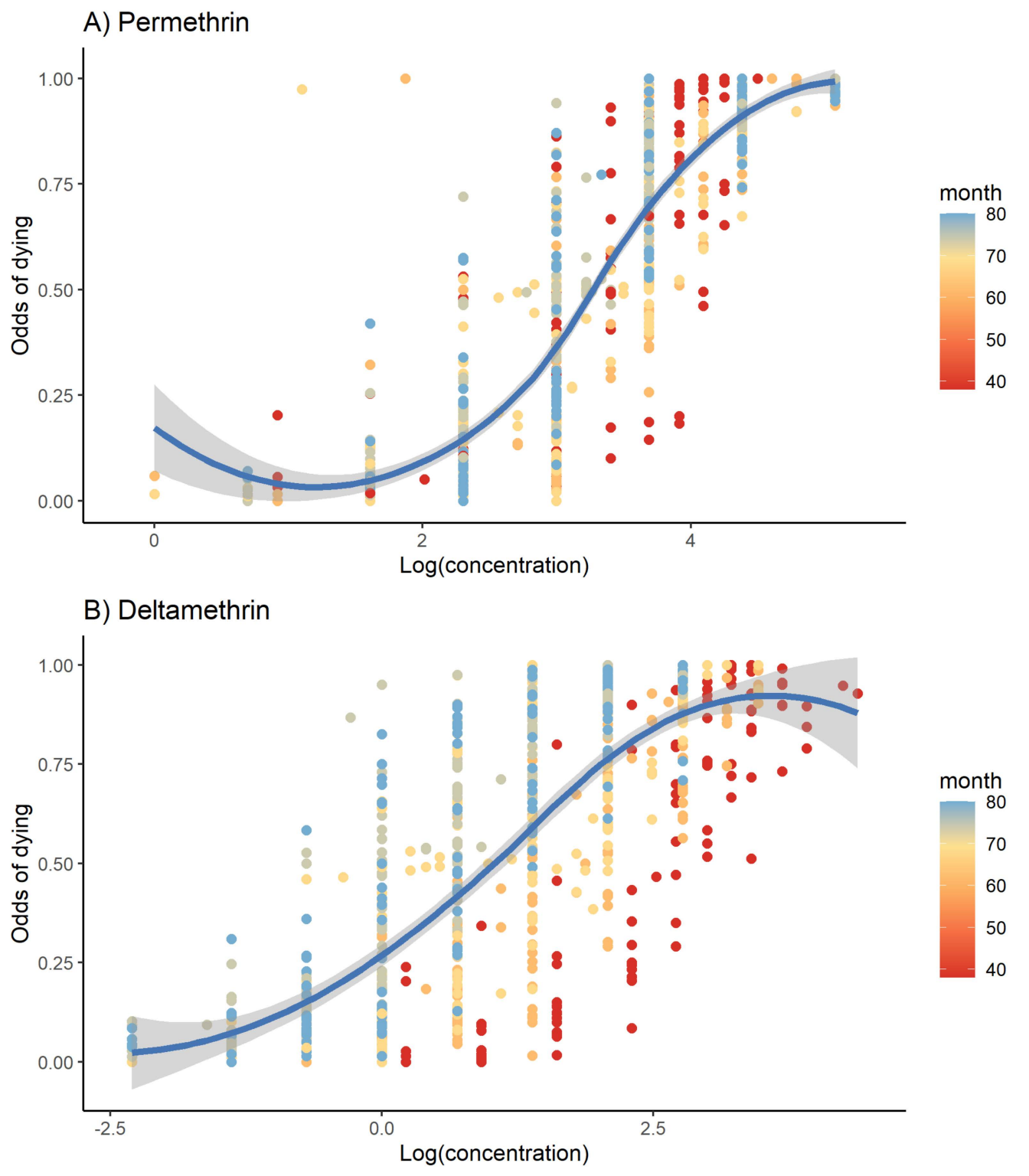
669 40. Gray L, Florez SD, Barreiro AM, Vadillo-Sanchez J, Gonzalez-Olvera G, Lenhart A, et al.
670 Experimental evaluation of the impact of household aerosolized insecticides on pyrethroid resistant
671 Aedes aegypti. Sci Rep. 2018;8(1):12535. Epub 2018/08/24. doi: 10.1038/s41598-018-30968-8. PubMed
672 PMID: 30135460; PubMed Central PMCID: PMCPMC6105583.
673 41. Smith LB, Silva JJ, Chen C, Harrington LC, Scott JG. Fitness costs of individual and combined
674 pyrethroid resistance mechanisms, kdr and CYP-mediated detoxification, in Aedes aegypti. PLoS Negl
675 Trop Dis. 2021;15(3):e0009271. Epub 2021/03/25. doi: 10.1371/journal.pntd.0009271. PubMed PMID:
676 33760828; PubMed Central PMCID: PMCPMC7990171.
677 42. Solis-Santoyo F, Rodriguez AD, Black W, Saavedra-Rodriguez K, Sanchez-Guillen D, Castillo-Vera
678 A, et al. Current enzyme-mediated insecticide resistance status of Aedes aegypti populations from a
679 dengue-endemic city in southern Mexico. Salud Publica Mex. 2023;65(1, ene-feb):19-27. Epub
680 2023/02/08. doi: 10.21149/13910. PubMed PMID: 36750076.
681 43. Campbell CL, Saavedra-Rodriguez K, Kubik TD, Lenhart A, Lozano-Fuentes S, Black WCt. Vgsc-
682 interacting proteins are genetically associated with pyrethroid resistance in Aedes aegypti. PLoS One.
683 2019;14(1):e0211497. Epub 2019/01/30. doi: 10.1371/journal.pone.0211497. PubMed PMID: 30695054;
684 PubMed Central PMCID: PMCPMC6350986.
685 44. Saavedra-Rodriguez K, Campbell CL, Lozano S, Penilla-Navarro P, Lopez-Solis A, Solis-Santoyo F,
686 et al. Permethrin resistance in Aedes aegypti: Genomic variants that confer knockdown resistance,
687 recovery, and death. PLoS Genet. 2021;17(6):e1009606. Epub 2021/06/18. doi:
688 10.1371/journal.pgen.1009606. PubMed PMID: 34138859.
689 45. Baltzegar J, Vella M, Gunning C, Vasquez G, Astete H, Stell F, et al. Rapid evolution of knockdown
690 resistance haplotypes in response to pyrethroid selection in Aedes aegypti. Evol Appl. 2021;14(8):2098-
691 113. Epub 2021/08/26. doi: 10.1111/eva.13269. PubMed PMID: 34429751; PubMed Central PMCID:
692 PMCPMC8372076.

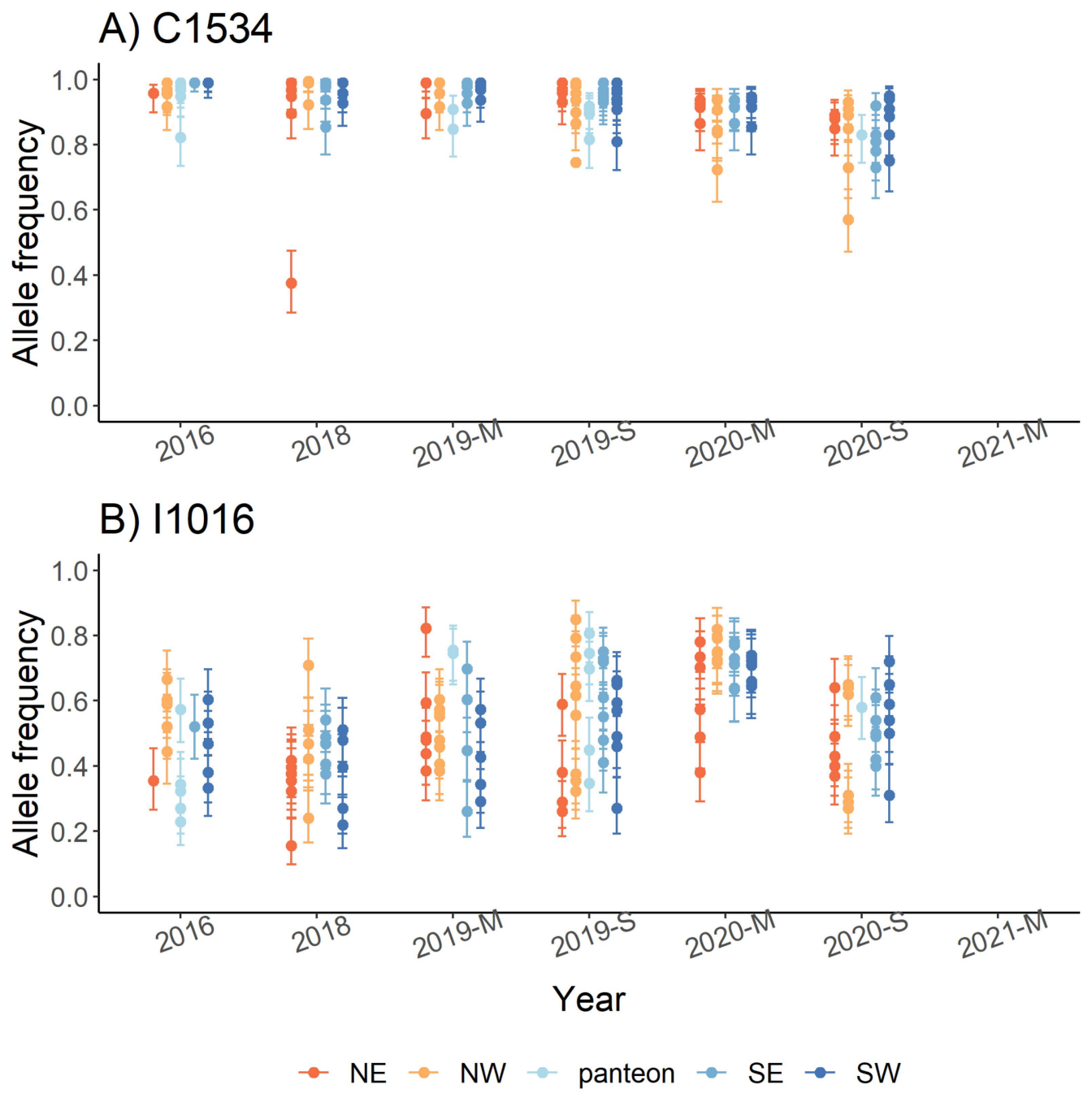
693

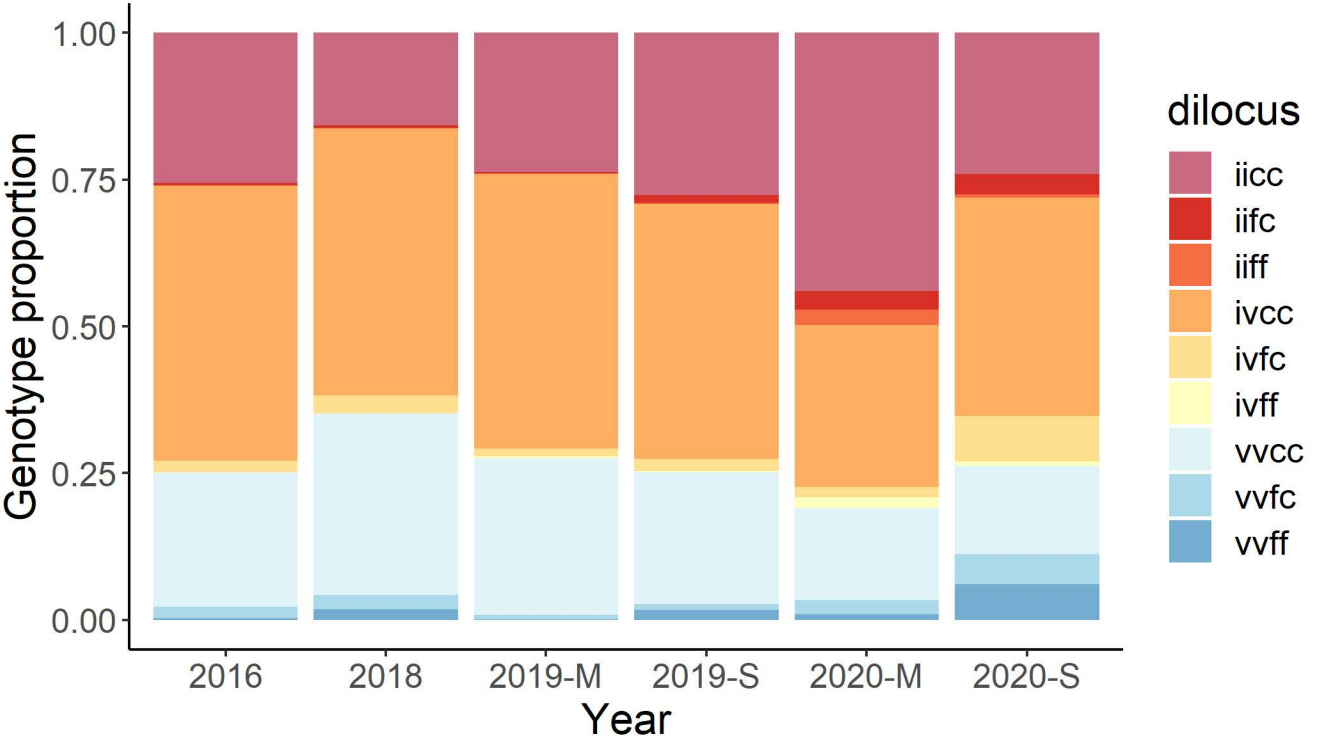

A) Whole-city spraying
Hectares
75000
50000
25000
0
2011
2012
2013
2015
2016
2017
2018
2019
2020
2021
2022
insecticide
Chlorpyrifos
Malathion
Phenothrin
Imida+Pra
B) Focalized spraying
Hectares
20000
15000
10000
5000
0
2011
2012
2013
2014
2015
2016
2017
2018
2019
2020
2021
2022
Year
insecticide
Chlorpyrifos
Malathion
Pirimiphos
Phenothrin
Deltamethrin
l-cyalothrin
Bifenthrin
Imida+Pra


Mexico
Tapachula
N
S
NW6
NW4
NW5
NW2
NW3
NW1
NE1
NE2
NE3
NE4
NE5
NE6
SW6
SE1
SE2
SE3
SE4
SE5
SE6
SW5
SW3
SW4
SW2
SW1


A) Permethrin
Resistance Ratio
75
50
25
2016
2018
2019-M
2019-S
2020-M
2020-S
2021-M
Year
B) Deltamethrin
Resistance Ratio
200
150
100
50
0
2016
2018
2019-M
2019-S
2020-M
2020-S
2021-M
Year
NE
NW
panteon
SE
SW


A) Permethrin
Odds of dying
1.00
0.75
0.50
0.25
0.00
0
2
4
Log(concentration)
month
80
70
60
50
40
B) Deltamethrin
Odds of dying
1.00
0.75
0.50
0.25
0.00
-2.5
0.0
2.5
Log(concentration)
month
80
70
60
50
40


A) C1534
Allele frequency
1.0
0.8
0.6
0.4
0.2
0.0
2016
2018
2019-M
2019-S
2020-M
2020-S
2021-M
B) I1016
Allele frequency
1.0
0.8
0.6
0.4
0.2
0.0
2016
2018
2019-M
2019-S
2020-M
2020-S
2021-M
Year
NE
NW
panteon
SE
SW


1.00
0.75
0.50
0.25
0.00
Genotype proportion
2016
2018
2019-M
2019-S
2020-M
2020-S
Year
dilocus
iicc
iifc
iiff
ivcc
ivfc
ivff
vvcc
vvfc
vvff

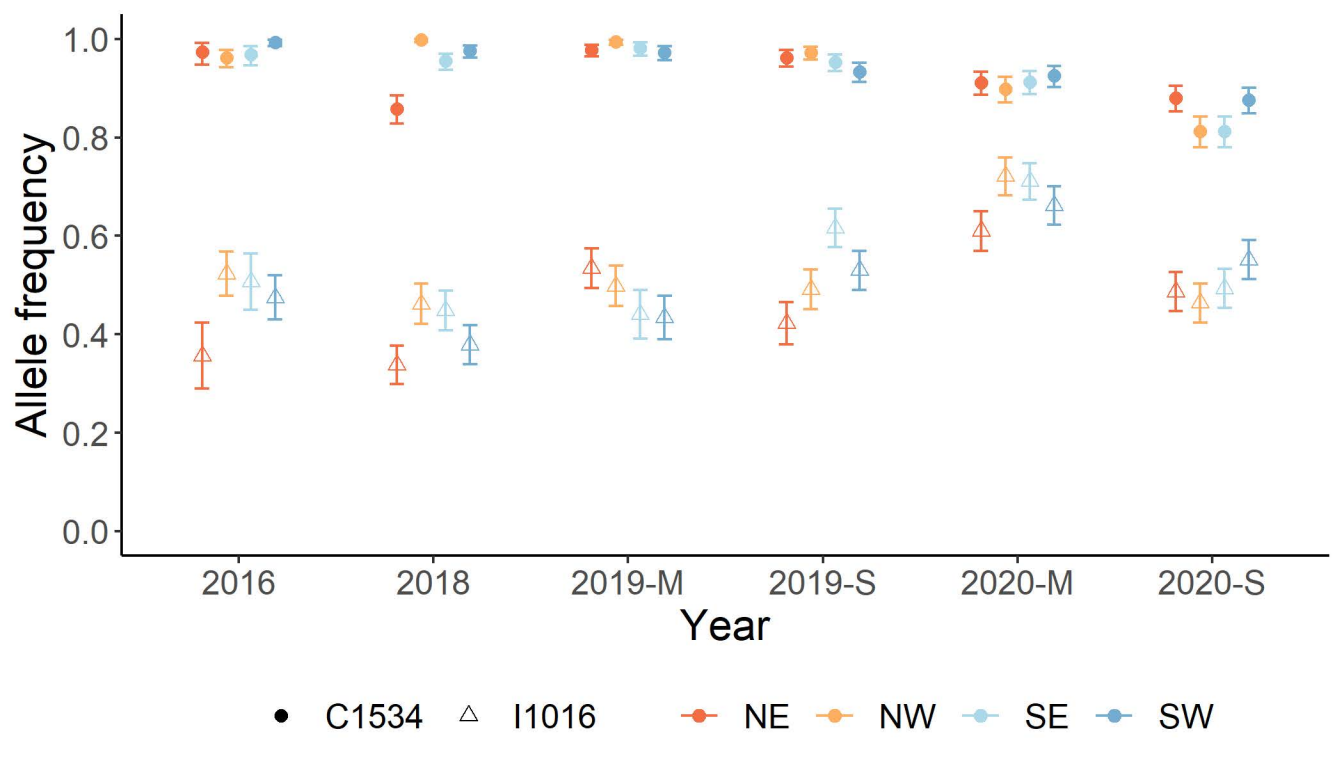

Allele frequency
1.0
0.8
0.6
0.4
0.2
0.0
2016
2018
2019-M
2019-S
2020-M
2020-S
Year
C1534
I1016
NE
NW
SE
SW

Table 1. Analysis of variance for multivariate logistic regression for permethrin and deltamethrin. The model tests the odds of dying as a response to insecticide concentration, time in months (after PYR discontinuations) and site (24 sites). The intercept, estimates and change in the odds of dying (%) for one unit of time (month) or one unit (log) of insecticide concentration holding other factors constant is shown.

Insecticide	**Variable**	**ANOVA**			**GLM regression estimates**		
		LR	Chisq	p value	Estimate	p value	**Change**
Permethrin	Intercept				-7.348	2.00E-16	
	Concentration	26314.7	1	2.2E-16	2.098	2.00E-16	814%
	Month	220.7	1	2.2E-16	0.015	2.00E-16	1.50%
	Site	1336.7	24	2.2E-16	-1.3 to 0.21	**	
Deltamethrin	Intercept				-6.781	2.00E-16	
	Concentration	22917	1	2.2E-16	1.498	2.00E-16	447%
	Month	5444.3	1	2.2E-16	0.0807	2.00E-16	8.40%
	Site	1528.7	24	2.2E-16	-0.95 to 0.6	‡ <0.005	

Df=degrees of freedom. ** 18 out of 24 site estimates were significant (<0.05), two of them were positive and 16 were negative. ‡ 10 out of 24 site estimates were significant.

Supplementary Table S1. Location of *Aedes aegypti* cemetery collections in the coast of Chiapas. Larvae were collected from each cemetery on 2016's wet season. The municipality name and geographical coordinates are provided.

Cemetery (panteon)	Municipality	Latitude	Longitude
Huixtla	Huixtla	15.136806	92.452194
Mapastepec	Mapastepec	15.445222	92.901222
Mazatan	Mazatan	14.867544	92.449967
Motozintla	Motozintla	15.365367	92.252292
Pijijiapan	Pijijiapan	15.684753	93.203253
Puerto Madero	Puerto Madero	14.722694	92.427417
Ciudad Hidalgo	Ciudad Hidalgo	14.685778	92.148889
Panteon Jardin	Tapachula	14.894917	92.249056
Panteon Municipal	Tapachula	14.90425	92.270306

Supplementary Table S2. Summary of generalized linear model estimates for A) Permethrin and, B) Deltamethrin. Independent variables include the insecticide concentration (log), site (24 sites), time in months (after PYR discontinuation). Estimates, standard error, z-value, probability and change are shown for each variable.

A) Permetrhin

Variable	Estimate	Std. Error	z value	Pr(>|z|)	Significance	Change
Intercept	-7.348	0.119	-61.779	< 2e-16	***	0.001
Log(concentration)	2.098	0.019	109.040	< 2e-16	***	8.148
Month	0.015	0.001	14.167	< 2e-16	***	1.015
Site_NE2	-1.196	0.100	-11.935	< 2e-16	***	0.302
Site_NE3	-0.074	0.104	-0.715	0.474877		0.929
Site_NE4	-0.772	0.105	-7.351	1.97E-13	***	0.462
Site_NE5	-0.582	0.100	-5.803	6.51E-09	***	0.559
Site_NE6	-0.966	0.097	-9.977	< 2e-16	***	0.381
Site_NW1	-0.826	0.096	-8.612	< 2e-16	***	0.438
Site_NW2	-1.372	0.084	-16.345	< 2e-16	***	0.254
Site_NW3	-0.430	0.093	-4.629	3.68E-06	***	0.651
Site_NW4	-0.824	0.098	-8.437	< 2e-16	***	0.438
Site_NW5	0.192	0.090	2.127	0.033394	*	1.212
Site_NW6	-0.599	0.096	-6.266	3.71E-10	***	0.550
Site_SE1	-0.659	0.100	-6.613	3.77E-11	***	0.517
Site_SE2	-0.072	0.100	-0.716	0.473898		0.931
Site_SE3	-1.125	0.085	-13.221	< 2e-16	***	0.325
Site_SE4	-0.664	0.102	-6.516	7.24E-11	***	0.515
Site_SE5	0.212	0.104	2.041	0.041232	*	1.236
Site_SE6	-0.203	0.098	-2.067	0.038763	*	0.816
Site_SW1	-0.115	0.083	-1.375	0.169204		0.892
Site_SW2	-0.047	0.093	-0.504	0.613916		0.954
Site_SW3	-0.404	0.097	-4.172	3.02E-05	***	0.668
Site_SW4	-0.321	0.098	-3.284	0.001024	**	0.726
Site_SW5	-1.255	0.103	-12.138	< 2e-16	***	0.285
Site_SW6	-0.284	0.085	-3.351	0.000805	***	0.753

B) Deltamethrin

Variable	Estimate	Std. Error	z value	Pr(>|z|)	Significance	Change
Intercept	-6.782	0.107	-63.178	< 2e-16	***	0.001
Log(concentration)	1.498	0.014	110.203	< 2e-16	***	4.474
Month	0.081	0.001	65.266	< 2e-16	***	1.084
Site_NE2	-0.951	0.087	-10.939	< 2e-16	***	0.387
Site_NE3	-0.272	0.091	-2.993	0.002761	**	0.762
Site_NE4	-0.594	0.098	-6.073	1.25E-09	***	0.552
Site_NE5	-0.836	0.091	-9.203	< 2e-16	***	0.434
Site_NE6	-0.944	0.088	-10.762	< 2e-16	***	0.389
Site_NW1	-0.535	0.086	-6.211	5.27E-10	***	0.586
Site_NW2	-0.312	0.077	-4.04	5.34E-05	***	0.732
Site_NW3	0.072	0.084	0.853	0.39366		1.075
Site_NW4	0.000	0.088	0.002	0.998245		1.000
Site_NW5	0.424	0.083	5.132	2.87E-07	***	1.528
Site_NW6	0.173	0.084	2.067	0.03875	*	1.189
Site_SE1	-0.930	0.090	-10.284	< 2e-16	***	0.394
Site_SE2	0.349	0.089	3.933	8.38E-05	***	1.417
Site_SE3	-0.799	0.077	-10.321	< 2e-16	***	0.450
Site_SE4	-0.309	0.090	-3.454	0.000553	***	0.734
Site_SE5	0.292	0.090	3.25	0.001154	**	1.338
Site_SE6	-0.185	0.093	-1.996	0.045898	*	0.831
Site_SW1	-0.489	0.077	-6.332	2.42E-10	***	0.613
Site_SW2	0.606	0.082	7.36	1.83E-13	***	1.833
Site_SW3	-0.706	0.085	-8.334	< 2e-16	***	0.493
Site_SW4	0.392	0.086	4.571	4.85E-06	***	1.481
Site_SW5	0.014	0.093	0.153	0.878695		1.014
Site_SW6	-0.114	0.077	-1.485	0.137452		0.892