Insecticide resistance in *Aedes aegypti* from Tapachula, Mexico: spatial variation and response to historical insecticide use

Spatial variation of insecticide resistance in *Aedes aegypti*

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24

25 Abstract

26 Background. Insecticide use continues as the main strategy to control *Aedes*
27 *aegypti*, the vector of dengue, zika, chikungunya, and yellow fever. In the city of
28 Tapachula, Mexico, mosquito control programs switched from pyrethroids to
29 organophosphates for outdoor spatial spraying in 2013. Additionally, the spraying
30 scheme switched from total coverage to focused control, prioritizing areas with
31 higher entomological-virological risk. Five years after this strategy had been
32 implemented, we evaluated the status and variability of insecticide resistance among
33 *Ae. aegypti* collected at 26 sites in Tapachula.

34 Methodology/Principal findings. We determined the lethal concentrations 50% of the
35 tested populations (LC_{50}) using a bottle bioassay, and then, we calculated the
36 resistance ratio (RR) relative to the susceptible New Orleans strain. Permethrin and
37 deltamethrin (pyrethroids), chlorpyrifos and malathion (organophosphates), and
38 bendiocarb (carbamate) were tested. The frequencies of the substitutions V1016I
39 and F1534C, which are in the voltage-gated sodium channel and confer knockdown-

resistance (*kdr*) to pyrethroid insecticides, were calculated. Despite 5 years having passed since the removal of pyrethroids from the control programs, *Ae. aegypti* remained highly resistant to permethrin and deltamethrin (RR > 10-fold). In addition, following 5 years of chlorpyrifos use, mosquitoes at 15 of 26 sites showed moderate resistance to chlorpyrifos (5- to 10-fold), and the mosquitoes from one site were highly resistant. All sites had low resistance to malathion (< 5-fold). Resistance to bendiocarb was low at 19 sites, moderate at five, and high at two. Frequencies of the V1016I ranged from 0.16– 0.71, while C1534 approached fixation at 23 sites (0.8–1). Resistance profiles and *kdr* allele frequencies varied across Tapachula. The variability was not associated with a spatial pattern at the scale of the sampling.

Conclusion/Significance. Mosquito populations respond to selection pressure at a focal scale in the field. Spatial variation across sites highlights the importance of testing multiple sites within geographical regions.

Keywords: *Aedes aegypti*; insecticide resistance; spatial variation; Mexico.

Excellent, the clarity and rigor of the manuscript is much improved. Nominal minor edits:

- 'Type I and II' typically precede pyrethroid (Line 331, 353-354)
- Please italicize kdr throughout
- Line 401: (37 Grossman) should be changed to [37].

Recommend acceptance

Author summary

Aedes aegypti is a major vector of dengue, chikungunya, zika, and yellow fever. In the absence of effective vaccines or treatments, the suppression of mosquito populations using insecticides commonly has been implemented by public health programs. Unfortunately, few insecticides are available to target adult mosquitoes with outdoor spraying. The mismanagement of insecticides can lead to resistance selection in mosquitoes, affecting our ability to control arboviral diseases. Ideally, screening insecticide susceptibility in local mosquito populations allows public health entities to support insecticide management strategies that will prevent the development of resistance. In this study, we evaluated insecticide resistance in *Ae. aegypti* collected across 26 sites in Tapachula, Mexico. The results reveal the response of populations to its historic use of insecticides. High resistance to pyrethroids, used for 10 years, but not in the previous 5 years, and moderate to high resistance to chlorpyrifos, an insecticide from a different toxicological group and used for the past 5 years, were confirmed. High variation in resistance across *Ae. aegypti* sites suggests that focal selection plays an important role in the evolution of insecticide resistance in the field. Screening several collections sites within a geographical region provides better evidence to support strategies of insecticide management.

Introduction

Aedes aegypti is the main vector of several arboviruses, including dengue, zika, chikungunya, and yellow fever. The control of this mosquito species is challenging, mainly because it is highly adapted to urban and suburban areas and because it is widely dispersed in endemic regions [1]. Except for yellow fever, safe and effective treatments or vaccines for these diseases are still under study. Therefore, the suppression of *Ae. aegypti* remains the cornerstone to prevent transmission and control of outbreaks of these diseases [2].

Effective vector control involves several strategies, such as the elimination of potential breeding sites, application of chemical insecticides, and implementation of biological control. However, the application of chemical insecticides has become a common form of control because such control is highly efficient and can be implemented promptly [3]. The most used insecticides in vector control have been the organophosphates temephos, used as a larvicide, and malathion, used as an adulticide by ultra-low volume application (ULV). Pyrethroids were introduced as adulticides in most Latin American countries in the 1990s [3]. In Mexico, according to the Mexican official policy for vector surveillance and control [4], the adulticide ULV formulation of permethrin, bioallethrin, and piperonyl butoxide (PBO) was used for more than 10 consecutive years (1999–2010). In the following 3–4 years, the pyrethroid d-phenothrin + PBO was introduced. Subsequently, the use of organophospates returned in 2013, with chlorpyrifos and malathion being used as adulticides, while carbamates were recommended for indoor residual application [5].

98 The prolonged use of pyrethroid insecticides resulted in the evolution of
99 resistance to them in *Ae. aegypti* worldwide, including Mexico, where failures in
100 dengue control strategies are due in part to resistance [5]. Given that resistance to
101 insecticides has been reported in populations of *Ae. aegypti* globally, the World
102 Health Organization (WHO) recommends testing to ensure an effective insecticide
103 management program. Decisions based on evidence of the resistance and/or
104 susceptibility of *Ae. aegypti* will ensure a better selection of insecticides in vector
105 control programs [6].

106 Two mechanisms of resistance to insecticides have been identified:
107 resistance due to the enhanced metabolism of insecticides and insensitivity at the
108 target site of the insecticides. Both mechanisms are involved in resistance to
109 pyrethroids [7]. Knockdown resistance (*kdr*) refers to a phenomenon in which insects
110 are not knocked down immediately after exposure to pyrethroids. *kdr* is caused by
111 specific mutations at the voltage-gated sodium channel (VGSC), which is the target
112 site for pyrethroids and DDT [8]. The amino acid substitutions V1016I [9], F1534C
113 [10], and V410L [11,12] frequently have been associated with resistance to
114 pyrethroids. Once these mutations are fixed in a population, reversion to
115 susceptibility is difficult to achieve [9]. Therefore, the detection and characterization
116 of *kdr* mutations in mosquito populations before resistance fixation occurs is
117 essential for insecticide management strategies.

118 In Mexico, Chiapas is one of the states with the highest rate of endemic
119 dengue cases. In particular, the city of Tapachula reports the highest incidence of
120 dengue in the state [13], which is attributed to the proliferation of vectors that transmit

121 emerging and re-emerging diseases. Under the region's tropical climate conditions,
122 *Ae. aegypti* maintains high densities throughout the year. Consequently, dengue and
123 other arboviruses transmitted by this vector have been prevalent in the region for a
124 long time [14].

125 In the context of insecticide resistance management, we investigated the
126 status of insecticide resistance to five insecticides, including two pyrethroids
127 (permethrin and deltamethrin), two organophosphates (chlorpyrifos and malathion),
128 and one carbamate (bendiocarb) and the spatial distribution of such resistance in
129 populations of *Ae. aegypti* throughout the city of Tapachula. We expect that after 5
130 years of heavy use of organophosphates and the removal of pyrethroids from vector
131 control campaigns, pyrethroid resistance will be lower, whereas organophosphate
132 resistance will appear in focal points of the city. We tested 26 collection sites located
133 in the city of Tapachula. Each collection site consisted of nine blocks and these were
134 selected based on vehicle access for outdoor spraying. To minimize the effects of
135 mosquito migration by flight range (50 - 150 m), sites were located at least 250 m
136 apart. The spatial correlation between resistance and geographical distance was
137 calculated for the 26 collection sites. In addition, since Tapachula's vector control
138 program uses a quadrant subdivision for spraying activities, we included a second
139 analysis to test this source of variation by assigning sites to one of the four cardinal
140 geographical quadrants (NE, NW, SE, and SW).

141

142 ## Materials and methods

143 **Collections.** The study was conducted in the city of Tapachula, Chiapas,
144 located in southern Mexico at 177 meters above sea level. The 26 collection sites
145 located in four quadrants in the city: Northwest (NW), Northeast (NE), Southwest
146 (SW), and Southeast (SE) are shown in Table 1. The biological material was
147 collected from January to April 2018 using ovitraps of 1-L capacity [15]. Twelve
148 ovitraps were installed at each collection site. Ovitraps were made by hand with
149 transparent, inert, non-toxic polypropylene (PP) cups of a 1 L capacity, and painted
150 black on the outside following the guidelines for Entomological Surveillance with
151 Ovitraps of the Mexican Ministry of Health (15). The interior of each ovitrap was lined
152 with Whatman filter paper (No. 1) and filled with water to ¾ capacity; the paper was
153 replaced weekly up to five times. The egg strips were transported to the insectary of
154 the Regional Center for Research in Public Health/National Institute of Public Health
155 (CRISP/INSP). The egg strips were submerged in 4 L of water in plastic containers
156 (40 cm x 30 cm x 15 cm). On the third and sixth day, the hatched larvae were fed a
157 diet of Harlan 5001 proteins, with 0.4 gr or 0.8 gr / 1.2 L for 1st-2nd stadium and 3rd-
158 4th stadium at the 3rd and 6th day, respectively.

159

160 **Table 1.** Geographic location of 26 *Aedes aegypti* collection sites in Tapachula,
161 Chiapas, Mexico, in 2018.

162

Quadrant	Site	Neighborhood	Abbreviation	Latitude	Longitude
Northeast					
	NE-1	Colinas del Rey	Col	14°55'50.9"	92°14'50.2"
	NE-2	Galaxias	Gal	14°55'11.2"	92°15"06.5"
	NE-3	Barrio Nuevo	Bar	14°54'51.0"	92°15'05.3"

	NE-4	San Juan de los Lagos	SJL	14°54'26.3"	92°15'13.4"
	NE-5	Coapantes	Coa	14°54'23.0"	92°14'57.1"
	NE-6	Bonanza	Bon	14°54'02.8"	92°14'31.7"
	NE-7	Centro (Country-Club)	CCC	14°54'22.7"	92°15'32.8"
Southeast					
	SE-1	Galeana	Gal	14°54'00.2"	92°15'56.0"
	SE-2	16 de Septiembre	16S	14°53'44.0"	92°15'42.1"
	SE-3	Calcáneo Beltrán	Cal	14°53'28.0"	92°15'43.4"
	SE-4	Benito Juárez 1	BJ1	14°53'21.8"	92°16'04.1"
	SE-5	Benito Juárez 2	BJ2	14°53'11.7"	92°16'10.3"
	SE-6	Emiliano Zapata	Zap	14°53'02.1"	92°16'14.2"
Southwest					
	SW-1	Raymundo Enríquez	Ray	14°52'01.4"	92°18'48.8"
	SW-2	Pobres Unidos	Pob	14°53'14.0"	92°17'6.1"
	SW-3	Palmeiras	Pal	14°53'22.1"	92°18'06.4"
	SW-4	Nuevo Milenio	Nue	14°53'24.8"	92°17'59.4"
	SW-5	Primavera	Pri	14°53'39.3"	92°17'38.6"
	SW-6	Democracia	Dem	14°54'23.7"	92°16'33.5"
Northwest					
	NW-1	5 de febrero	5Fe	14°55'33.7"	92°15'22.4"
	NW-2	Xochimilco 1	Xo1	14°55'48.9"	92°15'37.8"
	NW-3	Xochimilco 2	Xo2	14°56'02.2"	92°15'29.9"
	NW-4	Vergel 1	Ve1	14°56'21.2"	92°15'52.4"
	NW-5	Vergel 2	Ve2	14°56'32.9"	92°15'52.4"
	NW-6	Paraíso	Par	14°56'35.2"	92°15'19.7"
	NW-7	Centro (Nva. España)	CNE	14°54'35.0"	92°15'43.5"

Aedes aegypti mosquitoes were identified to species and placed in cages (30 cm^3). Females were bloodfed from rabbit to obtain the F_1 generation. Environmental conditions consisted of a temperature of 27 ± 2 °C, 70–80% humidity, and a 12:12 hour photoperiod. We used the insecticide-susceptible New Orleans reference strain of *Ae. aegypti*, provided by Dr. William Black and maintained in the CRISP/INSP insectary.

Bioassays. The F_1 adults were exposed to the insecticides using a modified CDC bottle bioassay (Centers for Disease Control) [16]. Sigma brand technical grade

172 insecticides were used to determine the lethal concentrations that killed 50% (LC_{50})
173 at each site. The pyrethroids permethrin (Type I) and deltamethrin (Type II), the
174 organophosphates malathion and chlorpyrifos, and the carbamate bendiocarb were
175 used to represent the toxicological groups used by vector control programs in
176 Mexico.

177 To determine the LC_{50}, we tested five to six insecticide concentrations, which
178 caused 10 to 90% mortality, in four replicates. Each insecticide LC_{50} required
179 approximately 500 mosquitoes. Table 2 shows the insecticide concentrations
180 (µg/bottle) used to coat 250 ml Wheaton bottles using acetone as the solvent. During
181 the bioassay, 15 to 20 (2-3 day old) females were gently aspirated into each bottle.
182 The knockdown effect was recorded every 10 minutes for 1 hour. After 1 hour of
183 exposure, the mosquitoes were transferred to plastic containers and maintained in
184 the insectary to observe the mortality at 24 hours. The LC_{50} of each insecticide was
185 also determined for the susceptible New Orleans reference strain (NO) using a
186 different set of insecticide concentrations (Table 2). Each insecticide LC_{50} was
187 replicated at least five times during a 7-month period. As control, a bottle
188 impregnated only with acetone was used each time a test with field or susceptible
189 mosquitoes was run.

190

191 **Table 2.** Concentrations (µg/bottle) used to determine the LC_{50} of five different
192 insecticides in the bottle bioassay for field *Aedes aegypti*.and the susceptible
193 reference strain.

Class	Mode of action	Insecticide	Concentration in µg/bottle	
			Field colonies	New Orleans reference
PYRs	sodium channel activators	Permethrin	10, 20, 40, 80, 160	0.8, 1.2, 2.4, 3.2, 6
		Deltamethrin	1, 2, 4, 6, 8, 16	0.75, 0.1, 0.15,0.2, 0.4
OPs	cholinesterase inhibitors	Malathion	2, 3, 4, 6, 8	2, 3, 4, 6, 8
		Chlorpyrifos	2, 4, 6, 8, 12	0.2, 0.4, 0.8, 1.6, 3.2
CARBs		Bendiocarb	0.5, 0.75, 1, 1.5, 3	0.25, 0.3, 0.4, 0.6, 1.2

194 PYRs = pyrethroids, OPs = organophosphates, CARBs = carbamates.

195 The LC_{50}, 95% confidence intervals, slope, intercept, and *p* values were
196 determined using the binary logistic regression model with QCal software [17]. The
197 null hypothesis (H_O) assumes the observed mortality curve adjusts to a binary logistic
198 regression model. Thus, we expected *p* values higher than 0.05 to accept the H_O.
199 When the Ho was rejected, the bioassay was repeated.

200 To estimate the level of resistance among sites, a resistance ratio (RR) was
201 calculated by dividing the LC_{50} of the field sites by the LC_{50} of the NO strain. The RR
202 criterion according to Mazzarri and Georghiou [18] classifies high resistance as RR
203 values greater than 10, moderate resistance as RR values between 5 and 10, and
204 low resistance as RR values less than 5.

205 **Genotyping *kdr*-associated mutations**. Genomic DNA was isolated from 50 F_1
206 individual female mosquitoes from each collection site following the method of Black
207 and DuTeau [19]. The DNA was resuspended in TE buffer (10 mM Tris-HCl, 1 mM
208 EDTA pH 8) and stored at -20 °C. The V1016I and F1534C mutations were
209 genotyped according to the protocols of Saavedra-Rodríguez et al. [9] and Yanola
210 et al. [10], respectively. The genotype and allelic frequencies were tested for Hardy-

211 Weinberg (HW) equilibrium. The null hypothesis is that equilibrium is present in the
212 population, which was verified with a chi-square test (df = 1 and p value > 0.05).

213 We tested the spatial variation of the LC_{50}s between the quadrants in the city
214 using a linear model and ANOVA in R (car package). To test the hypothesis of
215 resistance correlation with space, we created Moran's I correlograms as
216 implemented in PASSaGE 2.0 [20]. Mosquitoes from different collection sites were
217 considered neighbors if the sites were within 250 meters of each other. We expected
218 that the LC_{50}s or kdr frequencies would be associated with geographical distance
219 (i.e., that closer neighbor sites would show similar resistance levels, compared to
220 those farther away). A second analysis to test the variation of the LC_{50}s and kdr
221 frequencies between and within quadrants using a linear regression model and
222 ANOVA in R (car package) was conducted. Since the city is uniformly sprayed during
223 a cycle, we did not expect variation between or within quadrants. Correlation
224 between kdr frequencies and LC_{50}'s for permethrin and deltamethrin was tested
225 using a Spearman test.

226

227 ## Results

228 The geographic distributions of the resistance ratios (RR) for each insecticide
229 in the 26 sites in Tapachula are shown in Fig 1. The LC_{50} and confidence intervals
230 for each of the five insecticides are shown in Table S1. For the pyrethroids, we
231 observed high levels of resistance widespread across sites. Fig 2A shows the
232 permethrin RRs across Tapachula. High RRs were identified at 24 sites (RR from

11.4 to 43.1-fold). Only two sites—NE-3 and NW-2—showed moderate RRs (5.3 and 5.9-fold, respectively). The variation in RRs among quadrants was not significant ($F = 0.56$, df = 3, p value = 0.64). For deltamethrin, high RRs were determined in all 26 sites (10.6 to 101-fold). The variation among quadrants was not significant ($F = 1.08$, df = 3, p value = 0.37). Except for SW, all quadrants had at least one site with RR higher than 90-fold (Fig 2B).

Fig 1. Spatial distribution of insecticide resistance to five compounds in *Aedes aegypti* collected in Tapachula. The number above each bar corresponds to the resistance ratio (RR). The RR was calculated relative to the susceptible New Orleans reference strain. Map obtained from the National Institute of Statistics and Geography (INEGI). Digital Map of Mexico. MDM: http://gaia.inegi.org.mx/mdm6.

Fig 2. Pyrethroids resistance ratios (RRs) of *Aedes aegypti* collected in 26 sites across Tapachula in 2018. A) Permethrin and B) Deltamethrin. Dots represent the RR_{50} with 95% confidence intervals for each site. Horizontal lines indicate the threshold for low resistance (< 5-fold), moderate resistance (5- to 10-fold) and high resistance (> 10-fold).

The RRs for cholinesterase inhibitors (organophosphates and carbamates) are shown in Fig 3. For chlorpyrifos (Fig 3A), the RRs varied from low at 10 sites

254 (0.68- to 4.9-fold) to moderate at 15 sites (5.2- to 7.2-fold) to high at one site (10.2-
255 fold). No significant difference in RRs was found between quadrants (F = 1.08, df =
256 3, *p* value = 0.37). For malathion (Fig 3B), low resistance (0.86- to 4.5-fold) was
257 identified at all 26 sites. However, a significant difference was observed between
258 quadrants (F = 3.53, df = 3, *p* value = 0.03), with SE showing a mean RR of 2.6-fold
259 (95% CI 1.9- to 3.2-fold). Resistance to bendiocarb was low (1.2- to 4.8-fold) at 19
260 sites, moderate (7.3- to 9.9-fold) at five sites, and high (10.3- to 11.2-fold) at two
261 sites. No difference between quadrants was identified (F = 0.68. df = 3, *p* value =
262 0.57).

263

264 **Fig 3. Cholinesterase inhibitors resistance ratios (RRs) of *Aedes aegypti***
265 **collected in 26 sites across Tapachula in 2018.** A) Chlorpyrifos
266 (organophosphate), B) Malathion (organophosphate), and C) Bendiocarb
267 (carbamate). Dots represent the RR_{50} with 95% confidence intervals for each site.
268 Horizontal lines indicate the threshold for low resistance (< 5-fold), moderate
269 resistance (5- to 10-fold) and high resistance (> 10-fold).

270

271 ***Kdr*-associated mutations**

272 Genotype frequencies at the V1016I and F1534C loci in the voltage-gated
273 sodium channel gene were determined in a sample of 45-50 individuals from each
274 site (Table 3). The allele frequencies of the resistant allele I1016 fluctuated from

275 0.16–0.71. The lowest allele frequency (0.16) was scored for NE-3, whereas the
276 highest frequency was from NW-6 (0.71). The remaining sites ranged from 0.2 to
277 0.5. Except for NE-2 and SW-4, the genotype frequencies at the V1016I loci were
278 in HW equilibrium.

279 **Table 3.** Genotype counts and allele frequencies for two *kdr*-associated substitutions
280 (V1016I and F1534C) from *Aedes aegypti* collected at 26 sites in Tapachula. RR =
281 homozygote resistant, RS = heterozygote, and SS = homozygote susceptible. *
282 indicates a lack of Hardy-Weinberg equilibrium.

			V1016I genotypes				F1534C genotypes			
Site	Abv	N	I/I RR	V/I RS	V/V SS	I1016 frequency	C/C RR	F/C RS	F/F SS	C1534 frequency
NE-1	Col	48	4	23	21	0.32	47	1	0	0.99
NE-2	Gal	48	5	30	13	0.42*	39	8	1	0.90
NE-3	Bar	48	1	13	34	0.16	7	22	19	0.38
NE-4	SJL	48	6	22	20	0.35	47	1	0	0.99
NE-5	Coa	45	5	24	16	0.38	42	3	0	0.97
NE-6	Bon	48	8	22	18	0.4	43	5	0	0.95
NE-7	CCC	50	15	25	10	0.55	11	39	0	0.61*
	Subtotal	335	44	159	132	0.37	236	79	20	0.82*
SE-1	Gal	48	12	28	8	0.54	42	6	0	0.94
SE-2	16S	48	5	29	14	0.4	48	0	0	1*
SE-3	Cal	48	7	25	16	0.41	46	2	0	0.98
SE-4	BJ1	48	4	28	16	0.38	46	2	0	0.98
SE-5	BJ2	48	11	25	12	0.49	47	1	0	0.99
SE-6	Zap	48	10	25	13	0.47	34	14	0	0.85
	Subtotal	288	49	160	79	0.45	263	25	0	0.96
SW-1	Ray	48	5	28	15	0.39	48	0	0	1*
SW-2	Pob	48	14	21	13	0.51	47	1	0	0.99
SW-3	Pal	48	2	17	29	0.22	46	0	2	0.96*
SW-4	Nue	48	0	26	22	0.27*	48	0	0	1*
SW-5	Pri	48	8	22	18	0.39	47	1	0	0.99
SW-6	Dem	48	13	20	15	0.48	41	7	0	0.93
	Subtotal	288	42	134	112	0.38	277	9	2	0.98*
NW-1	5Fe	48	9	22	17	0.42	48	0	0	1*
NW-2	Xo1	48	10	29	9	0.51	48	0	0	1*

NW-3	Xo2	48	1	21	26	0.24	48	0	0	1*
NW-4	Ve1	48	11	23	14	0.47	48	0	0	1*
NW-5	Ve2	50	8	17	25	0.33	2	37	11	0.41*
NW-6	Par	48	24	20	4	0.71	48	0	0	1*
NW-7	CNE	50	4	26	20	0.34	0	38	12	0.38*
	Subtotal	340	67	158	115	0.43	242	75	23	0.82*
Total		1251	202	611	438	0.41	1018	188	45	0.89*

283

284 High allele frequencies of the resistant C1534 allele were determined at 22 of
285 the 26 sites, ranging from 0.85 to 1.0. Lower frequencies (0.38–0.41) were found in
286 NE-3, NW-5, and NW-7. While NE-7 was calculated with an intermediate value of
287 0.61. Most sites were in HW disequilibrium due to fixation of the resistant allele. We
288 conducted a Spearman correlation test between the pyrethroid LC_{50}s and the
289 expected frequencies of resistant homozygous genotypes. We found significant
290 correlation coefficients among permethrin LC_{50}s, I1016/I1016 homozygotes (S =
291 2588, rho = 0.53, *p* value = 0.002), and C1534/C1534 homozygotes (S = 1966, rho
292 = 0.515, *p* value = 0.004). Although it is known that C1534 shows protection only
293 against permethrin (12), for deltamethrin a significant correlation was observed
294 between the LC_{50} and I1016/I1016 homozygotes (S = 2643, rho = 0.467, *p* value =
295 0.008) and the C1534/C1534 homozygotes (S = 2945, rho = 0.507, *p* value = 0.002).
296 However, the significance for both insecticides disappeared when observations from
297 the New Orleans reference strain were removed.

298 To assess the correlation of LC_{50}'s with space, we generated Moran's I
299 correlograms for each of the five insecticides (Fig 4). The analysis included all 26
300 collection sites. We did not detect a discernable pattern in any of the tested

301 insecticides. We expected a positive correlation (Moran's I statistically > 0) between
302 nearby sites, then as the distance increased (between the samples) the correlation
303 would decrease, and later would turn negative (Moran's I statistically < 0). However,
304 this was not observed. Although, few of the distance classes where statistically
305 different from zero (eg. bendiocarb at 3250 m, 3750 m, 4750 m, and 6250 m;
306 malathion at 1500 m; and deltamethrin at 3750 m, and 4250 m). A caveat in our
307 analysis is the possibility that there is autocorrelation at smaller distances than the
308 ones we selected (x < 250 m). Our experimental design was not geared towards the
309 detection of spatial correlation at smaller distance; there is very small number of
310 samples below 250 meters.

311 **Fig 4. Moran's I correlograms as implemented in PASSaGE 2.0 assessing the**
312 **correlation of LC_{50}'s with space for permethrin (pyrethroid), deltamethrin**
313 **(pyrethroid), chlorpyrifos (organophosphate), malathion (organophosphate),**
314 **and bendiocarb (carbamate)**. The analysis included 26 collection sites in
315 Tapachula, Chiapas, Mexico. *Aedes aegypti* mosquitoes from different collection
316 sites were considered neighbors if the sites were within 250 meters of each other.

317

318 ## Discussion

319 Efforts to control *Ae. aegypti* populations are hindered by widespread
320 insecticide resistance worldwide. Local insecticide resistance monitoring is
321 necessary for the design of specific and successful resistance management
322 programs [21]. In Latin America, pyrethroids have been used for adult mosquito

323 control since the 1990s. The switch to pyrethroids was based on environmental
324 concerns that led to the use of less toxic classes of insecticides [22]. In Mexico,
325 vector control programs implemented the use of permethrin in 1999 and continued
326 their use until 2010. Local selection pressure caused a rapid evolution of pyrethroid
327 resistance in *Ae. aegypti* across Mexico [9,23-27], resulting in policy modifications
328 that recommended the use of insecticides with different toxicological modes of
329 action.

330 In Tapachula, vector control programs replaced the use of permethrin by a
331 different pyrethroid Type I (d-phenothrin + piperonyl butoxide) from 2010 to 2013. In
332 2013, pyrethroids were replaced by the organophosphate chlorpyrifos, and in 2017,
333 by malathion. This study reveals the current status and response of local *Ae. aegypti*
334 populations to these insecticide shifts. Despite the switch to organophosphates in
335 the last 5 years, we observed that high levels of pyrethroid resistance remain
336 widespread in Tapachula. An assumption in insecticide resistance management is
337 that insecticide resistance has negative fitness costs. Therefore, when insecticide
338 pressure is removed, populations are expected to reverse to susceptibility [28,29].
339 Currently, we are conducting a study to determine the degree of loss of resistance
340 to pyrethroids from 2016 to 2020 in this study area, which will demonstrate whether
341 mosquito populations in Tapachula are undergoing a process of decreasing
342 resistance that will take several years. Another explanation is that pyrethroid
343 resistance is maintained in *Ae. aegypti* populations by the domestic use of
344 pyrethroids [30]. Surveys in Merida, Mexico, indicate that 85% of households took

345 action to kill pests, and 89% exclusively targeted mosquitoes. Interestingly most of
346 the aerosol spray cans contained pyrethroid insecticides [31].

347 Interestingly, RRs for deltamethrin—a Type II pyrethroid—were higher than
348 permethrin RRs across sites. Deltamethrin was authorized by CENAPRECE for
349 indoor residual use in 2009 for control of the malaria vector, but its use was restricted
350 to rural areas. Therefore, direct selection pressure from the use of deltamethrin in
351 public health is unlikely to be responsible for the high RRs in *Ae. aegypti* from
352 Tapachula. Although all pyrethroids act at the same target site, the variability of
353 resistance to their different types is attributed to different binding sites for pyrethroids
354 Type I and Type II at the voltage-gated sodium channel. Additionally, the presence
355 of enzymes that have a greater affinity to metabolize specific molecules within the
356 same toxicological group might explain this variability [32].

357 Knockdown resistance (*kdr*) is a major mechanism of pyrethroid resistance in
358 *Ae. aegypti* from Mexico. In this study, we measured the frequency of this
359 mechanism by molecular tests that identify mutations that confer changes to amino
360 acids in the VGSC. The allele frequencies of the resistant allele I1016 ranged from
361 0.4 to 0.7, and for the resistant allele C1534, from 0.85 to 1.0 (except for three sites
362 that had ~0.4). Historical data of kdr mutations indicated that C1534 confers low level
363 of resistant on its own, and that resistance increased dramatically when I1016
364 evolved from the V1,016/C1,534 haplotype in field mosquito collected in different
365 places from Mexico (33). Those results demonstrated that I1016 was unlikely to have
366 evolved independently, and that both mutations need coexist in the same mosquito
367 in order to confer higher levels of resistance. Moderate correlations were significant

368 between the resistant allele frequencies and the RRs for permethrin and
369 deltamethrin only when including the New Orleans datapoints. This significance
370 might be explained by most of the allele frequencies being distributed within a small
371 range of variability.

372 This study was conducted after chlorpyrifos had been used for 5 years in
373 outdoor spraying by vector control programs. Our results provide evidence of the
374 response of *Ae. aegypti* populations to chlorpyrifos pressure. Ten sites showed low
375 RRs, 15 sites showed moderate resistance, and one site was highly resistant.
376 Interestingly, *Ae. aegypti* from all 26 sites were susceptible or had low RRs to
377 malathion, thereby indicating that resistance to chlorpyrifos does not predict the lack
378 of effectivity of malathion. Additionally, the RRs to bendiocarb were variable:
379 mosquitoes from 19 sites had low RRs, those from three were moderate, and those
380 from two were highly resistant. Only a few sites showed moderate to high resistance
381 to both chlorpyrifos and bendiocarb (NE-5, NW-6, and SE-4). The lack of cross-
382 resistance between organophosphates and carbamates suggests that the resistance
383 mechanisms are not due to the insensitivity of their target site (the
384 acetylcholinesterase) [34] that in fact no mutations have been found in *ace-1* gene
385 in *Aedes aegypti* [35].

386 A survey in Veracruz, Mexico, identified high RRs to chlorpyrifos in
387 Cosoleacaque (RR = 13.9), moderate RRs in Poza Rica (RR = 7.9), and low RRs in
388 five sites in Veracruz [36]. By using a discriminating dose of 50 µg/bottle and 85
389 µg/bottle for 30 minutes, two additional studies were able to identify chlorpyrifos
390 resistance in Mexico [26,37]. Since neither of these studies found a history of

391 chlorpyrifos use in vector control programs, the resistance might be explained by
392 indirect exposure to chlorpyrifos through the extensive use of this insecticide to
393 control agricultural pests [36].

394 During vector control programs, the city of Tapachula is uniformly sprayed,
395 using the same insecticide, frequency and intensity. More yet, we selected sites
396 based in their accessibility to spraying-vehicles. Assuming that no spatial
397 heterogeneity in frequency and intensity of spraying, we did not expect the high
398 levels of variation in resistance profiles across ~~de~~ the city. For example, significant
399 heterogeneity in the frequency of kdr haplotypes was detected in *Ae. ~~aegytpi~~* aegypti
400 collected between city blocks in a town of Yucatan, suggesting that selection for
401 these haplotypes occurs at a fine spatial scale ~~(37 GROSSMAN)~~ [37]. However, in
402 contrast to our study, insecticide application was highly variable in space and time,
403 creating a mosaic of selection pressures. In our study, some sources of
404 heterogeneity could occur from mosquito migration from untreated sites due to
405 vehicle inaccessibility, including parks, cemeteries, steep and unpaved streets. A
406 second source of insecticide pressure is by use of household aerosol insecticides.
407 For example, in a previous study from Merida, Mexico approximately 87% of
408 households used commercially available pyrethroid products to control mosquitoes
409 in their homes. Future studies should include an assessment of this source of
410 selection pressure in Tapachula.

411 The spatial variability in insecticide resistance observed across the 26 sites in
412 Tapachula is likely associated ~~to~~ with the presence or appearance of "hot spots or dengue
413 foci," which contribute to the persistent transmission of the diseases and therefore

414 to focal areas with greater spray intervention [38]. In addition, the spatial variability
415 of resistance highlights the importance of evaluating resistance in multiple sites
416 within a defined geographic area for the application of appropriate vector control
417 decisions. Although no geographical correlation/association/pattern between
418 resistance was found in Tapachula, more specific and finer environmental
419 characteristics must not be discarded in future studies. A previous study used
420 mitochondrial ND4 haplotypes to determine gene flow patterns among 38 *Ae.*
421 *aegypti* coastal collections in Mexico (39). Three genetic clusters were identified, the
422 Northeast, Pacific, and Yucatan peninsula. For all sites, genetic distances remained
423 small below geographic distances of 90 km and became large at distances >150 km.
424 The Pacific cluster had the highest gene flow and diversity. A second study in the
425 Yucatan Peninsula showed high gene flow occurring across 27 *Ae. aegypti* collection
426 sites located up to 150 km of distance. Single nucleotide polymorphism (SNPs) at
427 eleven loci did not vary across sites, suggesting high levels of gene flow. In contrast,
428 insecticide resistance loci, including kdr alleles (I1016 and C1534) were highly
429 variable across sites, indicating that insecticide resistance offsets the homogenizing
430 effects of gene flow (40). In this study, we assume complete gene flow among
431 collection sites because: 1) Tapachula belongs to the Pacific cluster, 2) *Ae. aegypti*
432 is well established throughout the year and, 3) collection sites are within 10 km of
433 distance. However, this remains to be tested.

434

435 ## Conclusion

Despite more than 5 years having passed since the removal of pyrethroids from vector control programs in Tapachula, high levels of pyrethroid resistance and *kdr*-associated alleles persist in *Ae. aegypti* populations. Future resistance surveys will reveal if pyrethroid resistance is maintained in mosquito populations. We observed that, after 5 years of chlorpyrifos use in vector control programs, more than 50% of the sites have moderate to high chlorpyrifos resistance but complete susceptibility to malathion. Since malathion was introduced later in 2017, future studies to evaluate the selection of malathion resistance in the field are needed. Two different analyses were conducted 1) the spatial analysis included all 26 sites and, 2) the quadrant analysis to identify operational sources of heterogeneity. The quadrant analysis doesn't include a geographical component and has limitations. Insecticide resistance varied spatially, most likely as a consequence of the pattern of insecticide use combined with environmental factors. Based on the results of our study, we suggest that both of the studied organophosphates and the carbamate remain viable options for use in the control strategy for this vector. The return to a pyrethroid (at least permethrin and deltamethrin) for outdoor spraying is recommended when the levels of resistance have decreased to RR less than 10-fold and once mechanisms other than *kdr* have been elucidated for pyrethroid resistance.

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Supplementary Tables

Table S1. Lethal concentration to kill 50% (LC_{50}) for five insecticides at 26 *Aedes aegypti* sites in Tapachula, Chiapas, Mexico. The LC_{50} is in micrograms (µg) of active ingredient per bottle. The 95% confidence intervals around the LC_{50} are enclosed in parentheses. *p* values higher than 0.05 indicate the observed data fit the binary logistic regression model.

Table S2. Genotype for two *kdr*-associated substitutions (V1016I and F1534C) per individual *Aedes aegypti* mosquito (n = 47 to 50) at 26 sites in Tapachula, Chiapas, Mexico. For V1016I: AA = homozygote for Ile_{1016}, resistant; GG = homozygote for Val_{1016}, susceptible; AG = heterozygote Ile_{1016}/Val_{1016}. For F1534C: TT homozygote for Phe_{1534}, susceptible; GG = homozygote for Cys_{1534}, resistant; TG = heterozygote Phe_{1534}/Cys_{1534}.

Figure 1

Click here to access/download;Figure;Fig 1 corrected_PACE.tif

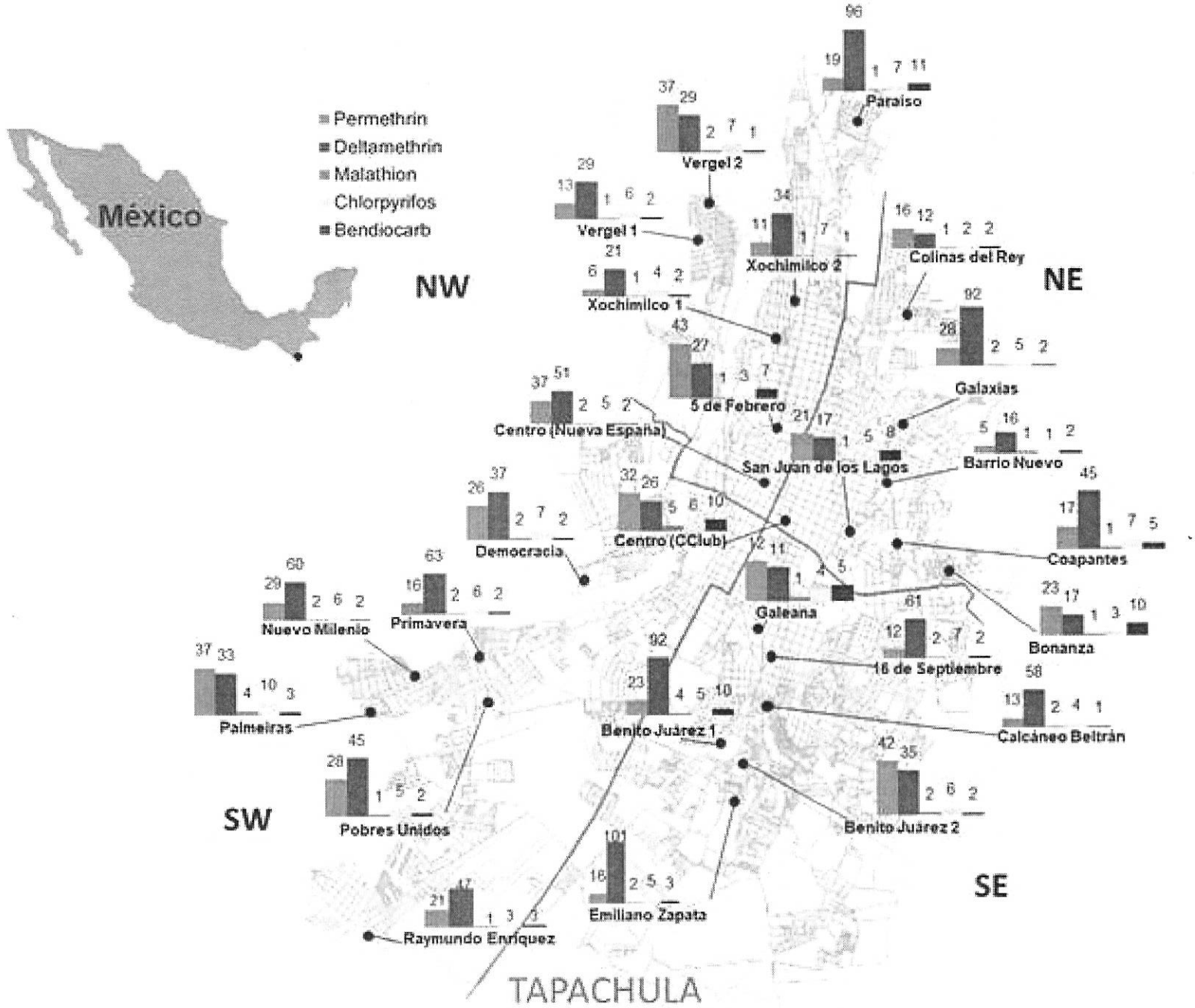

Figure 2

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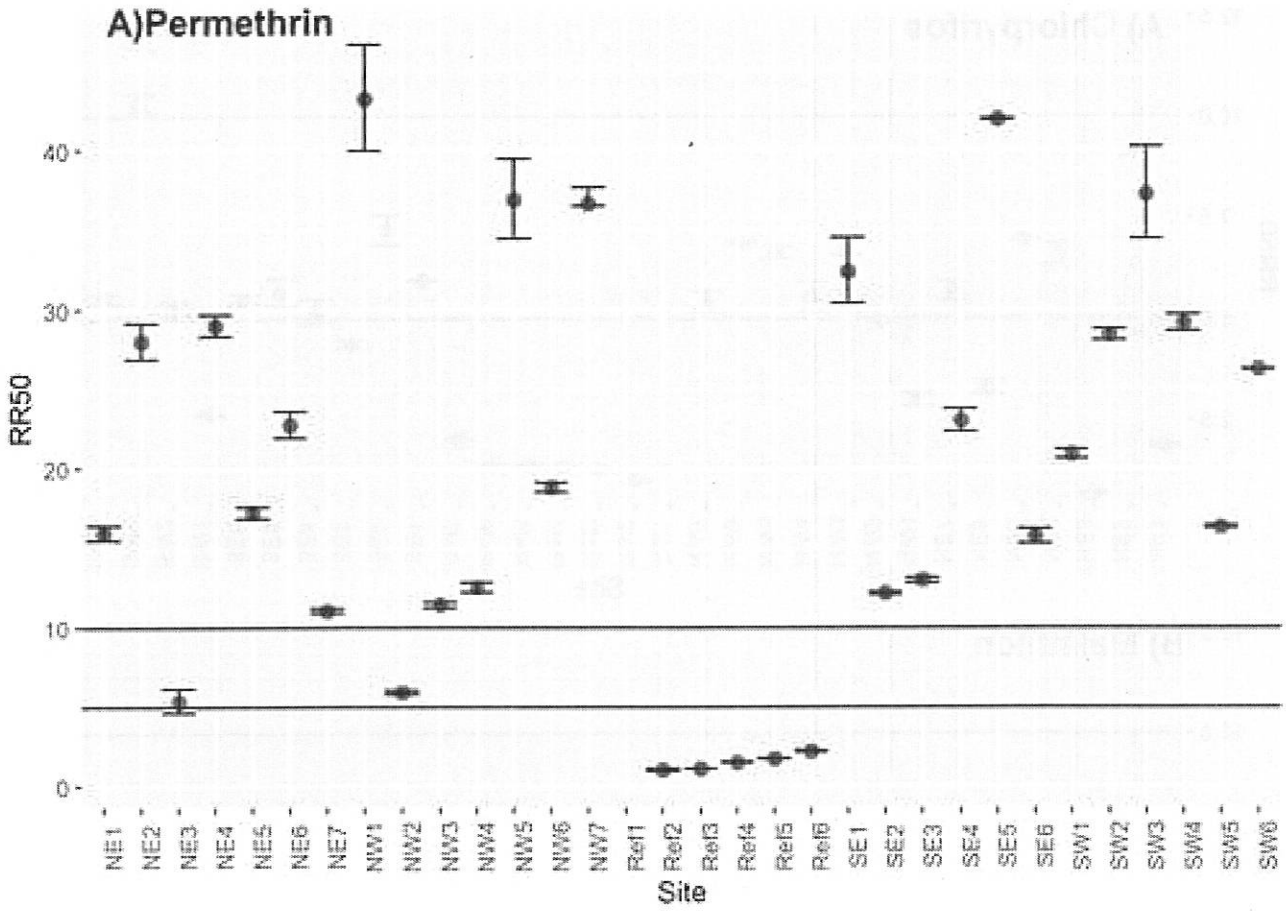

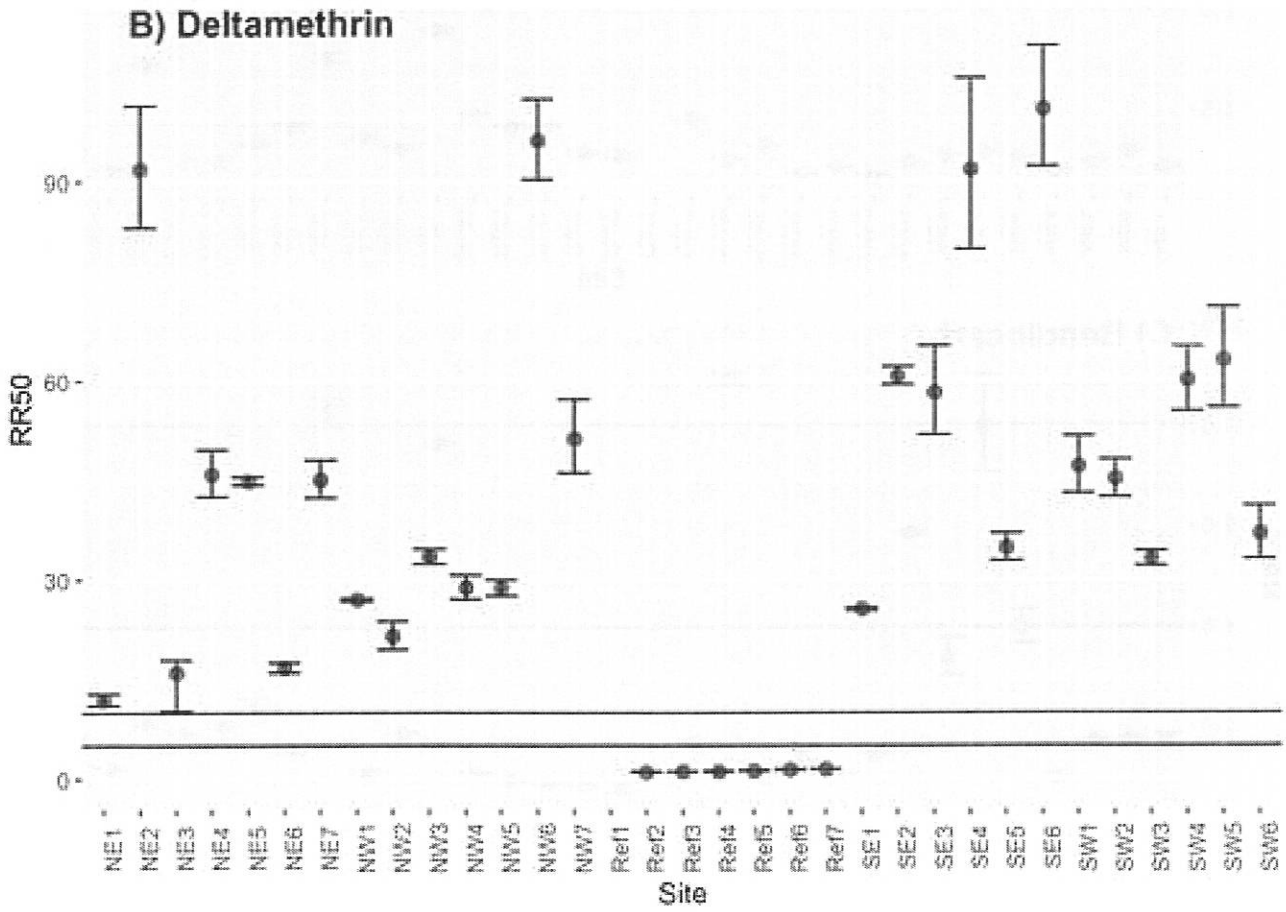

Figure 3

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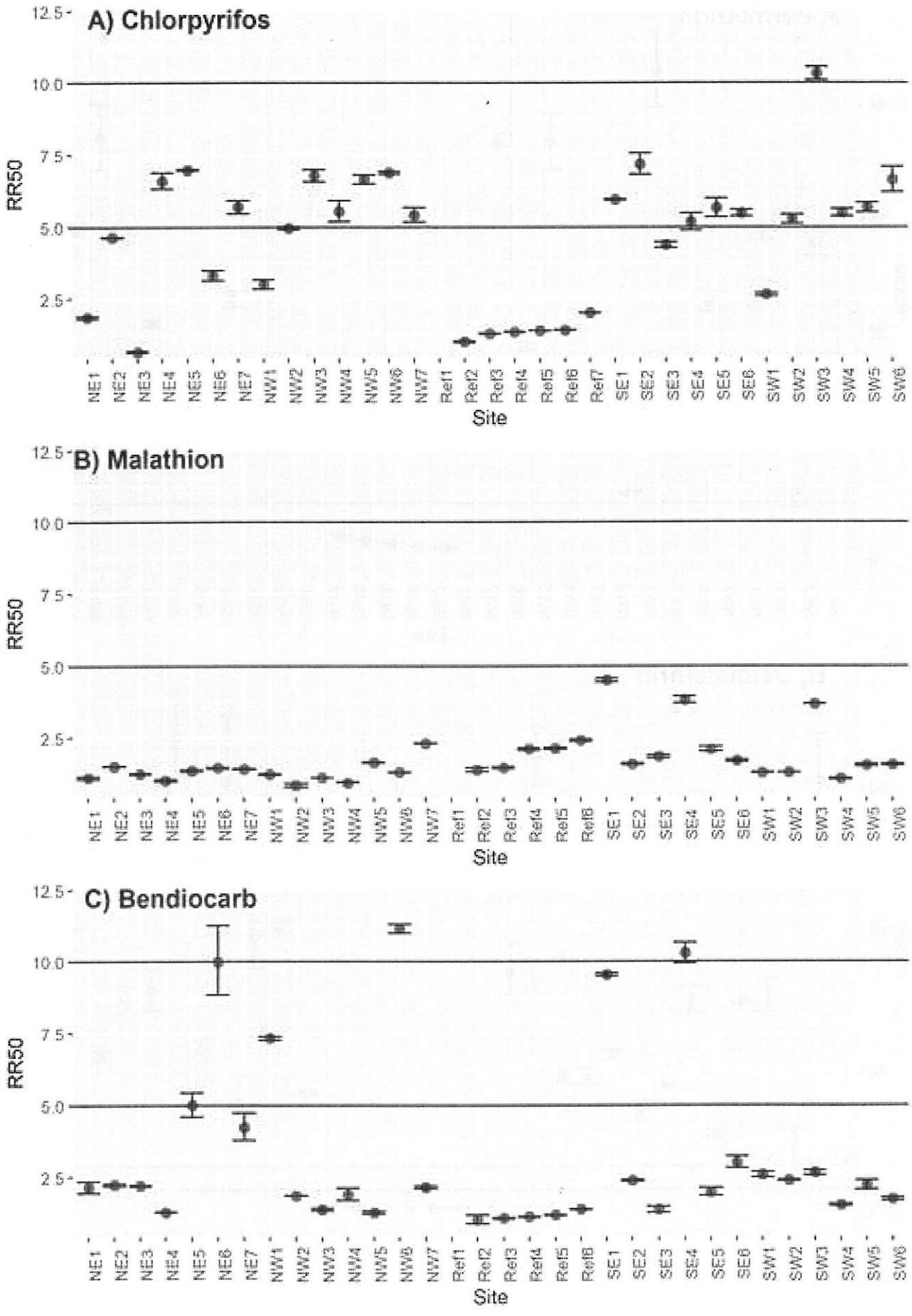

Figure 4

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