Current enzyme-mediated insecticide resistance status of *Aedes aegypti* populations from a dengue-endemic city in southern Mexico

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Abstract

Objective. To identify the enzyme-mediated insecticide resistance in *Aedes aegypti* in Tapachula, Mexico. **Materials and methods.** Biochemical assays were undertaken to determine the enzyme levels in mosquitoes from 22 sites collected in 2018 and 2020 in Tapachula. Results of 2018 were correlated with the resistance to insecticides published. **Results.** Mosquitoes had higher levels than those of the susceptible strain in 2018 and 2020 respectively of α-esterases in 15 and 12 sites; β-esterases in 7 and 6 sites; glutathione-S-transferases in 11 and 19 sites; ρNPA-esterases in 21 and 17 sites; and cytochromes P^{450} in 20 and 22 sites. In mosquitoes of 2018, there was a moderate correlation between previously documented Malathion resistance ratios and the insensitive acetylcholinesterase (r=0.459, *p*= 0.03). **Conclusions.** The elevated enzyme levels found indicate

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Resumen

Objetivo. Identificar la resistencia a insecticidas mediada por enzimas en *Aedes aegypti* de Tapachula, México. **Material y métodos.** Se realizaron ensayos bioquímicos para calcular los niveles enzimáticos en mosquitos de 22 sitios colectados en Tapachula en 2018 y 2020. Resultados de 2018 se correlacionaron con la resistencia a insecticidas publicada. **Resultados.** Se obtuvieron niveles más altos que los de la cepa susceptible en 2018 y 2020, respectivamente, de: α-esterasas en 15 y 12 sitios; β-esterasas en 7 y 6 sitios; glutatión-S-transferasas en 11 y 19 sitios; ρNPA-esterases en 21 y 17 sitios; y citocromos P^{450} en 20 y 22 sitios. Los índices publicados de resistencia al malatión y la acetilcolinesterasa insensible tuvieron una correlación moderada (r=0.459, *p*= 0.03) en mosquitos de 2018. **Conclusiones.** Los altos niveles enzimáticos encontrados indican su contribución en

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its contribution to the resistance to pyrethroids and organophosphates already published in mosquitoes from Tapachula. Bioassays using enzyme inhibitors resulted in greater mortality, confirming that metabolism contributes to resistance.

Keywords: insecticide resistance; enzymes; metabolism; acetylcholinesterase; *Aedes aegypti*

la resistencia a piretroides y organofosforados en mosquitos de Tapachula de 2018. Bioensayos con inhibidores enzimáticos dieron mayores mortalidades, confirmando que el metabolismo contribuye en la resistencia.

Palabras clave: resistencia a insecticidas; enzimas; metabolismo; acetilcolinesterasa; *Aedes aegypti*

Aedes aegypti L. (*Diptera: Culicidae*), vector of dengue, Zika, chikungunya, and yellow fever, is a mosquito species with a nearly global distribution; except for yellow fever, none of the other diseases have a vaccine. Epidemiological surveillance and vector control have been crucial in preventing and reducing the transmission of these diseases.[1]

Aedes aegypti is widespread throughout Mexico and the prevalence of the four dengue serotypes maintains endemic and hyperendemic transmission.[2] Chemical control has been the most widely used method in vector prevention and control programs; consequently, targeted applications of adulticide insecticides such as pyrethroids (PYR), organophosphates (OP), and carbamates (CARB) are carried out, while larvicides such as temephos (OP), novaluron (benzoylurea), and spinosad (macrolide) are used to eliminate larval breeding sites.[3] In addition to the limited number of chemicals available for the control of *Aedes aegypti*, insecticide resistance among mosquito populations has been a cause of concern. Insecticide resistance is known to be primarily mediated by two types of mechanisms: the first due to changes in the target site of insecticides, such as the knockdown resistance (*kdr*) mechanism, which mutations occur in the sodium channel regulated by voltage from the nervious cells, reported in PYR resistant populations of *Ae. aegypti* and *Ae. albopictus*,[4,5] and in the acetylcholinesterase (AChE) which mutations in *Anopheles* render resistance to the insecticides OP and CARB.[6] The second mechanism is mediated by genome modifications that control the expression of enzymes involved in the detoxification of insecticides or alter their affinity to insecticides.[7] Esterases,[8,9] glutathione-S-transferases (GST),[10] and cytochromes P450[11,12] are typically related with resistance to PYRs,[13,14] although esterases also are associated with resistance to OPs and CARB.[15]

In endemic regions, mosquitoes are under constant insecticide pressure. In Mexico, the vector control program applies OP and CARB in "transmission hot spots" where disease transmission is maintained.[16] Thus, mosquito populations have responded to selection pressure at a focal scale in the field, as was observed in most of the 26 collection sites of *Ae. aegypti* populations made in 2018 in Tapachula, where low to moderate resistance to OP and CARB was reported, while they were still highly resistant to PYR.[17]

This work is part of a research project which aim was to identify the resistance and mechanisms in *Ae. aegypti*, and how they responded to the historical use of insecticides in Tapachula from 2018 to 2021, where mosquito were collected twice a year in 26 sites. Preliminary results of the insecticide resistance diagnosis for the first year collected is already published,[17] while the insecticide resistance behavior thorough the years is being prepared for publication. Here was investigated whether *Ae. aegypti* populations have a resistance mediated by overexpressed enzymes and whether their acetylcholinesterase is insensitive (AChEi) to OP and CARB. For this objective, biochemical assays were undertaken in two mosquito samples of the same collection sites, one at the beginning (2018) and another at the half of the study (2020). Additionally for the year 2018, whether the resistance to insecticides already reported[17] correlates with the enzyme results was investigated in the same mosquito populations.

Materials and methods

Study sites

Tapachula, Chiapas is located in southern Mexico on the border with Guatemala; it is a cosmopolitan city because of its high immigration activity, which has likely made it endemic to dengue fever with outbreaks occurring throughout the year. Aedes aegypti were collected from 22 sites in Tapachula[17] between January to April of 2018 and 2020 using 12 ovitraps per site (figure 1).[18] The ovitrap consisted of a one-liter plastic container coated with a 15 cm-wide strip of #615 filter paper that was replaced weekly for four continuous months in order to collect sufficient eggs. The biological material was sent to the *Centro Regional de Investigación en Salud Pública* / *Instituto Nacional de Salud Pública* (CRISP/INSP) in order to produce adult mosquitoes. Emerged mosquitoes were identified to species following the Rueda´s method (2004).[19] Mosquitoes were housed in

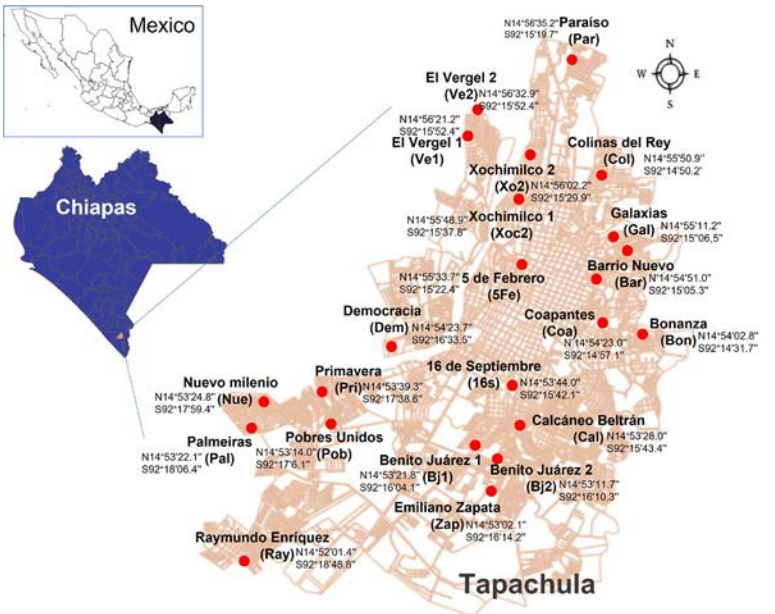

FIGURE 1. SPATIAL DISTRIBUTION OF 22 SITES FROM TAPACHULA WHERE *AEDES AEGYPTI* WAS COLLECTED FOR THE EZYME-MEDIATED INSECTICIDE RESISTANCE STUDY. MAP OBTAINED FROM THE *INSTITUTO NACIONAL DE ESTADÍSTICA Y GEOGRAFÍA*[18]

an insectary at 27 ± 2°C, 70 to 80% humidity, and 12:12 photoperiod of light-to-dark. The susceptible strain New Orleans (N.O.) (mosquitoes susceptible to insecticides used by the dengue vector control program and tested here) was used as a reference colony and maintained under identical conditions. This research project was evaluated by the INSP ethics committee.

Biochemical assays

The biochemical assays were conducted according to Penilla and colleagues.[20] Two batches of 47 three-day-old female mosquitoes were individually homogenized in 200 μl of sterile distilled water and distributed in 47 of 96 wells of Corning #3590 plate. Duplicates of 25 μl of each homogenate were dispensed onto a 96-well microplate for the AChE assay. The remaining homogenates were centrifuged at 4 000 rpm/4°C/30 min, and the supernatants were distributed in duplicates into microplates placed on ice for α- and β- esterases (20 μl), ρ-nitrophenyl acetate (ρNPA)- esterases (10 μl), GST (10 μl), cytochromes P^{450} (20 μl), and proteins (10 μl). Each microplate had two control wells with water instead of the homogenate. All enzymatic reactions were measured using a microplate reader Multiskan spectrum.

AChE assay

Two microplates were prepared using 25 μl of the homogenate, 145 μl of phosphate-Triton buffer (Triton X-100 at 1% in 0.1M sodium phosphate buffer, pH 7.8), and 10 μl of DTNB solution (dithiobis-2-nitrobenzoic acid 0.01 M in 0.1 M sodium phosphate buffer, pH 7.0). One of the duplicates included 25 μl of ASCHI substrate (Acetylthiocholine iodide 0.01M) while the other contained 25 μl of ASCHI substrate containing 0.2% propoxur 0.1 M. The kinetics of the enzymatic reaction was continuously monitored at 405 nm for 5 min, after which the inhibition percentage of AChE by propoxur relative to the unfettered wells was determined.

α- and β- esterase assays

The first of two supernatant duplicates of 20 μl was to calculate the amount of α-esterases while the second of β-esterases. The first duplicate received 200 μl of the sodium α-naphthyl acetate solution (100 μl of 30 mM α-NA in acetone in 10 ml of 0.02M phosphate buffer, pH 7.2), while the second received 200 μl of the sodium β-naphthyl acetate solution. The reaction was halted by adding 50 μl of the fast blue dye solution (22.5 mg of

fast blue in 2.25 ml of distilled water and 5.25 ml of 5% sodium lauryl sulfate diluted in 0.1M sodium phosphate buffer pH 7.0) after 30 min at room temperature. The reaction product was measured at a wavelength of 570 nm at a fixed point. The results were reported in nmol of product generated per min/mg of protein.

ρNPA-esterases assay

Into both duplicates of 20 μl of each supernatant, 200 μl of the substrate ρNPA (ρ-nitrophenyl acetate 100 mM in acetonitrile, and 50 mM phosphate buffer, pH 7.4, 1:100) were added. ρNPA activity per individual was reported in μmol/min/mg of protein using an extinction coefficient of 6.53 mM^{-1} (corrected for a path length of 0.6 cm), and a wavelength of 405 nm for 2 min.

Glutathione S-transferase assay

Each duplicate containing 10 μl of supernatant was mixed with 200 μl of GSH/CDNB (10 mM reduced glutathione prepared in 0.1M phosphate buffer, pH 6.5, and 63 mM chlorodinitrobenzene diluted in methanol). Enzyme kinetics was measured at 340 nm for 5 min. GST activity per individual was reported in mmol of conjugated CDNB/min/mg of protein, corrected for the length of the volume and using an extinction coefficient of 9.6 mM^{-1} cm^{-1}.[21]

Cytochrome P^{450} assay

Each duplicate containing 20 μl of supernatant was first mixed with 80 μl of potassium phosphate buffer (0.0625 M, pH 7.2). Then, 200 μl of TMB solution (0.01g of 3,3,5,5-tetramethylbenzidine diluted in 5 ml of methanol and mixed with 15 μl of 0.25M sodium acetate buffer, pH 5.0) were added, plus 25 μl of the substrate (5% hydrogen peroxide). The reaction was incubated at room temperature for two hours and measured at a wavelength of 650 nm at a fixed point. The optical density of each individual mosquito was compared to the standard curve of known concentrations of cytochrome P^{450} 2B4 from rabbit. The results were reported in pmol of cytochromes P^{450}/mg of protein.

Protein assay

Each 10 μl duplicate of the homogenate was mixed with 300 μl of the Bio-Rad solution (Dye Reagent Concentrated, BioRad)[22] in a 1:4 dilution with distilled water. Following a 5 min incubation at room temperature, the reaction was measured at 570 nm. Each mosquito's protein concentration was determined and compared to the standard curve derived from the bovine serum albumin.

The mean activity or enzymatic content of *Ae. aegypti* populations from each site was compared to that of N.O. and between years using the Anova, Kruskal-Wallis and Dunnet's test, with a significance of 95%. The correlation between the enzymatic activities and the resistance ratios (RR) reported in 2018 was tested with a Spearman test using the IBM SPSS Statistics v.26.

Bioassay with synergists of PYR

Following the CDC recommendation for synergists tests, batches of 240 to 300 4-5-day-old female mosquitoes from Col site were exposed in bottles per an hour each to Permethrin, to Permethrin + Piperonyl butoxide (PBO), to Permethrin + S,S,S-Tributyl phosphorotrithioate (DEF), and to Permethrin + Diethyl maleate (DM), and mortality documented after 24 hours. A set of susceptible mosquitoes from the N.O. strain was also exposed to the insecticide and synergists.

Results

AChE

Compared to a susceptible strain, individuals with an AChE inhibition percentage by propoxur below 60% were considered resistant.[23] In our mosquito populations, the inhibition percentages by propoxur were significantly lower at 18 of 22 sites in 2018 (those of Bar being the lowest), and in 20 of 22 sites in 2020 (those of Pob being the lowest) compared to those of the N.O. ($p<0.0001$, tables I and II). In 2018, mosquitoes from Bj1, Pob, Ve2, and Par in 2018, and Cal and Bj2 in 2020, did not differ significantly from the N.O. The sites of Col in 2018 and Pob in 2020 had the highest number of mosquitoes with an inhibition percentage < 60% (table III).

α- and β- esterases

In year 2018, mosquitoes from 15 of 22 sites had considerably high levels of α -esterases ($p<0.0001$), with Coa mosquitoes having the highest levels (table I, figure 2). In 2020, the number of sites with higher levels of α-esterases than N.O. was reduced to 12, with 5Feb having the highest levels (figure 2 and table II).

In 2018, seven of 22 sites (Coa, Bon, 16S, Ray, Dem, Xo1 and Par) showed β-esterases levels significantly higher than those of the susceptible ($p<0.001$) (table I, figure 2). In 2020, only 6 of the 22 sites (Gal, Coa, 16S, Bj2 Dem and 5Fe) had greater levels than the susceptible ($p<0.0001$) (table II).

Table I
MEANS AND STANDARD DEVIATIONS (±SD) OF ENZYME LEVELS IN *AE. AEGYPTI* COLLECTED FROM 22 SITES IN TAPACHULA, CHIAPAS, MEXICO, IN 2018

Sites	*AChE inhibition percentages*	α-esterases	β-esterases	*Glutathion S-transferases*	ρNPA-esterasas	*Cytochromes P450*
Col	56.65 ± 14.57*	0.00029 ± 0.00012*	0.00012 ± 0.00008	0.44 ± 0.29	0.073 ± 0.028*	0.0030 ± 0.0010*
Gal	60.96 ± 15.73*	0.00034 ± 0.00019*	0.00015 ± 0.00010	1.42 ± 1.17*	0.089 ± 0.044*	0.0028 ± 0.0013*
Bar	56.18 ± 15.01*	0.00018 ± 0.00011	0.00009 ± 0.00006	1.61 ± 1.65*	0.609 ± 0.618*	0.0031 ± 0.0014*
Coa	60.39 ± 20.07*	0.00057 ± 0.00036*	0.00033 ± 0.00028*	0.60 ± 0.31	0.146 ± 0.095*	0.0032 ± 0.0009*
Bon	62.84 ± 12.18*	0.00039 ± 0.00023*	0.00020 ± 0.00010*	0.36 ± 0.23	0.094 ± 0.028*	0.0030 ± 0.0011*
16S	65.96 ± 17.48*	0.00048 ± 0.00018*	0.00028 ± 0.00020*	1.67 ± 1.99*	1.539 ± 0.678*	0.0045 ± 0.0042*
Cal	66.71 ± 15.77*	0.00028 ± 0.00014*	0.00006 ± 0.00004	0.49 ± 0.36	0.077 ± 0.051*	0.0042 ± 0.0013*
Bj1	70.30 ± 14.42	0.00030 ± 0.00014*	0.00017 ± 0.00008	2.21 ± 1.92*	0.691 ± 0.798*	0.0037 ± 0.0013*
Bj2	59.11 ± 16.10*	0.00027 ± 0.00014*	0.00012 ± 0.00010	0.76 ± 0.663	0.634 ± 0.702*	0.0041 ± 0.0016*
Zap	64.69 ± 14.56*	0.00021 ± 0.00010	0.00013 ± 0.00009	0.76 ± 0.39	0.072 ± 0.029*	0.0028 ± 0.0007*
Ray	58.14 ± 19.22*	0.00039 ± 0.00031*	0.00032 ± 0.00030*	1.87 ± 1.94*	0.367 ± 0.361*	0.0056 ± 0.0037*
Pob	68.20 ± 17.39	0.00014 ± 0.00009	0.00009 ± 0.00008	0.63 ± 0.39	0.085 ± 0.036*	0.0048 ± 0.0018*
Pal	83.03 ± 8.49*	0.00011 ± 0.00007	0.00005 ± 0.00004	1.46 ± 0.63*	0.085 ± 0.028*	0.0029 ± 0.0015
Nue	67.87 ± 14.46*	0.00032 ± 0.00020*	0.00009 ± 0.00006	1.97 ± 0.82*	0.080 ± 0.058*	0.0063 ± 0.0023*
Pri	63.23 ± 12.27*	0.00021 ± 0.00010	0.00009 ± 0.00007	0.49 ± 0.27	0.055 ± 0.038	0.0035 ± 0.0012*
Dem	57.96 ± 15.47*	0.00047 ± 0.00029*	0.00026 ± 0.00016*	0.49 ± 0.37	0.088 ± 0.041*	0.0030 ± 0.0013*
5Fe	65.19 ± 14.25*	0.00018 ± 0.00009	0.00006 ± 0.00003	1.90 ± 1.61*	0.089 ± 0.038*	0.0038 ± 0.0007*
Xo1	65.30 ± 14.44*	0.00051 ± 0.00042*	0.00036 ± 0.00035*	0.59 ± 0.340	0.099 ± 0.040*	0.0034 ± 0.0017*
Xo2	61.66 ± 16.67*	0.00023 ± 0.00010*	0.00007 ± 0.00004	0.51 ± 0.31	0.092 ± 0.028*	0.0031 ± 0.0007*
Ve1	57.15 ± 19.20*	0.00022 ± 0.00016	0.00010 ± 0.00006	1.13 ± 0.64*	0.266 ± 0.212*	0.0044 ± 0.0011*
Ve2	71.36 ± 20.12	0.00021 ± 0.00013*	0.00008 ± 0.00005	0.96 ± 0.45*	0.704 ± 0.819*	0.0037 ± 0.0009*
Par	68.11 ± 15.21	0.00043 ± 0.00021*	0.00022 ± 0.00013*	2.06 ± 1.60*	0.090 ± 0.034*	0.0023 ± 0.0012
Total		0.00032 ± 0.00023	0.00016 ± 0.00018	1.10 ± 1.21	0.2627 ± 0.4784	0.0037 ± 0.00195
N.O.	75.80 ± 8.96	0.000165 ± 0.00009	0.00014 ± 0.00005	0.32 ± 0.22	0.050 ± 0.024	0.0018 ± 0.0008

* Significantly higher than the average for the New Orleans strain ($p<0.05$). AChE: Acetylcholinesterase, ρNPA: ρ-Nitrophenyl Acetate (ρNPA). Values for α- and β esterases are nmol of the product formed (α- or β- Naphthol Acetate)/min/mg of protein; values for ρNPA-esterases are μmol of the product formed (ρ-Nitro Phenol Acetate)/min/mg of protein; Values for Glutathion S-transferases are mmol of conjugated CDNB/min/mg of protein; Values of Cytochrome P^{450} are pmol of Cytochromes P^{450}/mg of protein.

ρNPA- esterases

In 2018, mosquitoes from 21 sites had higher enzyme activity than from N.O. (table II), with the greatest values observed in 16S. Only Pri mosquitoes did not differ significantly from N.O. mosquitoes (figure 2). While in 2020, only 17 sites had significant higher levels than those of the N.O. (p-0.0001) (table II), with the greatest levels in those of 5Fe (figure 2).

Glutathione S-transferases

In 2018, high activity of GST were detected at 11 out of 22 sites compared to N.O. ($p<0.001$) (table I and figure 2). For the year 2020, the number of sites with significantly higher levels than those of N.O. ($p<0.001$) increased to 19, with Bar with the highest values (table II and figure 2).

Cytochromes P^{450}

In 2018, mosquitoes at 20 sites had significantly higher levels than N.O. ($p<0.0001$) (table I). Only mosquitoes from Par and Pal did not differ (figure 2) from N.O. In 2020, mosquitoes from all sites had significantly higher levels than those of the N.O. ($p<0.001$) (table II), with Pal having the highest levels (figure 2). The mosquitoes from 16S and Ray in 2018 and Gal, Dem, and 5Fe in 2020 had the greatest amounts of all enzymes.

Table II

Means and standard deviations (±SD) of enzyme levels in *Ae. aegypti* collected from 22 sites in Tapachula, Chiapas, Mexico, in 2020

Sites	*AChE inhibition percentages*	α-esterases	β-esterases	*Glutathion S-transferases*	ρNPA-esterasas	*Cytochromes P450*
Col	67.34 ± 13.1*	0.00034 ± 0.00022*	0.00011 ± 7.8E-05	0.57± 0.31*	0.168± 0.050*	0.0041± 0.0014*
Gal	56.02 ± 14.5*	0.00044 ± 0.00017*	0.00020 ± 1.3E-04*	0.44± 0.26*	0.197± 0.062*	0.0043± 0.0019*
Bar	54.50 ± 17.2*	0.00022 ± 0.00014	0.00006 ± 3.8E-05	1.22± 0.93*	0.185± 0.052*	0.0029± 0.0011*
Coa	55.01 ± 12.8*	0.00040 ± 0.00025*	0.00017 ± 1.3E-04*	0.42± 0.25*	0.141± 0.046	0.0032± 0.0018*
Bon	67.66 ± 9.9*	0.00029 ± 0.00013	0.00007 ± 4.8E-05	0.45± 0.25*	0.136± 0.037	0.0035± 0.0014*
16S	67.53 ± 14.7*	0.00039 ± 0.00020*	0.00022 ± 1.4E-04*	0.36± 0.19	0.137± 0.057	0.0021± 0.0008*
Cal	73.34 ± 10.1	0.00029 ± 0.00011	0.00012 ± 7.1E-05	0.53± 0.28*	0.189± 0.047*	0.0041± 0.0030*
Bj1	59.34 ± 13.4*	0.00035 ± 0.00015*	0.00012 ± 9.2E-05	1.14± 0.93*	0.207± 0.059*	0.0036± 0.0017*
Bj2	76.25 ± 8.4	0.00028 ± 0.00012	0.00017 ± 9.8E-05*	0.44± 0.20*	0.138± 0.047	0.0023± 0.0011*
Zap	66.44 ± 13.5*	0.00034 ± 0.00023*	0.00014 ± 1.2E-04	0.38± 0.29	0.196± 0.084*	0.0052± 0.0049*
Ray	69.90 ± 8.7*	0.00030 ± 0.00012*	0.00007 ± 3.7E-05	0.46± 0.27*	0.141± 0.040	0.0020± 0.0014*
Pob	52.00 ± 12.1*	0.00026 ± 0.00012	0.00008 ± 5.0E-05	0.48± 0.26*	0.168± 0.037*	0.0039± 0.0016*
Pal	63.12 ± 12.1*	0.00021 ± 0.00011	0.00005 ± 4.3E-05	0.43± 0.26*	0.256± 0.073*	0.0054± 0.0032*
Nue	61.42 ± 10.2*	0.00025 ± 0.00010	0.00009 ± 6.6E-05	0.50± 0.32*	0.205± 0.059*	0.0035± 0.0024*
Pri	67.70 ± 16.3*	0.00030 ± 0.00010*	0.00008 ± 4.1E-05	0.51± 0.23*	0.184± 0.041*	0.0026± 0.0009*
Dem	62.22 ± 9.3*	0.00035 ± 0.00011*	0.00024 ± 1.3E-04*	0.37± 0.18*	0.195± 0.047*	0.0037± 0.0012*
5Fe	60.10 ± 13.8*	0.00047 ± 0.00019*	0.00018 ± 8.7E-05*	0.44± 0.24*	0.468± 0.329*	0.0031± 0.0019*
Xo1	55.36 ± 22.2*	0.00034 ± 0.00015*	0.00010 ± 7.4E-05	0.46± 0.22*	0.167± 0.039*	0.0031± 0.0016*
Xo2	64.61 ± 10.9*	0.00022 ± 0.00012	0.00009 ± 6.6E-05	0.40± 0.27*	0.167± 0.044*	0.0044± 0.0025*
Ve1	59.36 ± 16.5*	0.00027 ± 0.00017	0.00012 ± 8.5E-05	0.38± 0.22*	0.210± 0.062*	0.0037± 0.0029*
Ve2	63.53 ± 13.4*	0.00036 ± 0.00015*	0.00008 ± 5.0E-05	0.37± 0.26	0.350± 0.221*	0.0031± 0.0014*
Par	62.67 ± 12.3*	0.00025 ± 0.00012	0.00016 ± 1.1E-04	0.52± 0.36*	0.181± 0.046*	0.0046± 0.0031*
Total		0.00032 ± 0.00017	0.00013 ± 0.00010	0.511 ± 0.43	0.199 ± 0.123	0.0036 ± 0.00233
N.O	77.82 ± 6.4	0.00022 ± 0.00015	0.00008 ± 00007	0.26± 0.14	0.113± 0.077	0.0013± 0.0009

* Significantly higher than the average for the New Orleans strain ($p<0.05$). AChE: Acetylcholinesterase, ρNPA: ρ-Nitrophenyl Acetate (ρNPA). Values for α- and β esterases are nmol of the product formed (α- or β- Naphthol Acetate)/min/mg of protein; values for ρNPA-esterases are μmol of the product formed (ρ-Nitro Phenol Acetate)/min/mg of protein; Values for Glutathion S-transferases are mmol of conjugated CDNB/min/mg of protein; Values of Cytochrome P^{450} are pmol of Cytochromes P^{450}/mg of protein.

Table III

Number of mosquitoes with inhibition percentages of AChE by propoxur less than 60% from 22 populations of *Aedes aegypti* from Tapachula, collected in 2018 and 2020

Collection year/site	*Col*	*Gal*	*Bar*	*Coa*	*Bon*	*16S*	*Cal*	*Bj1*	*Bj2*	*Zap*	*Ray*
2018	52	31	48	38	31	23	25	16	40	28	41
2020	19	52	54	60	17	36	4	36	4)	27	13
Collection year/site	*Pob*	*Pal*	*Nue*	*Pri*	*Dem*	*5Fe*	*Xo1*	*Xo2*	*Ve1*	*Ve2*	*Par*
2018	26	2	20	29	40	25	27	37	38	26	25
2020	66	29	32	24	39	38	34	31	40	22	30

n=94, except Bj1(2020) and Par(2020) n=93, Pal(2018) n=47.

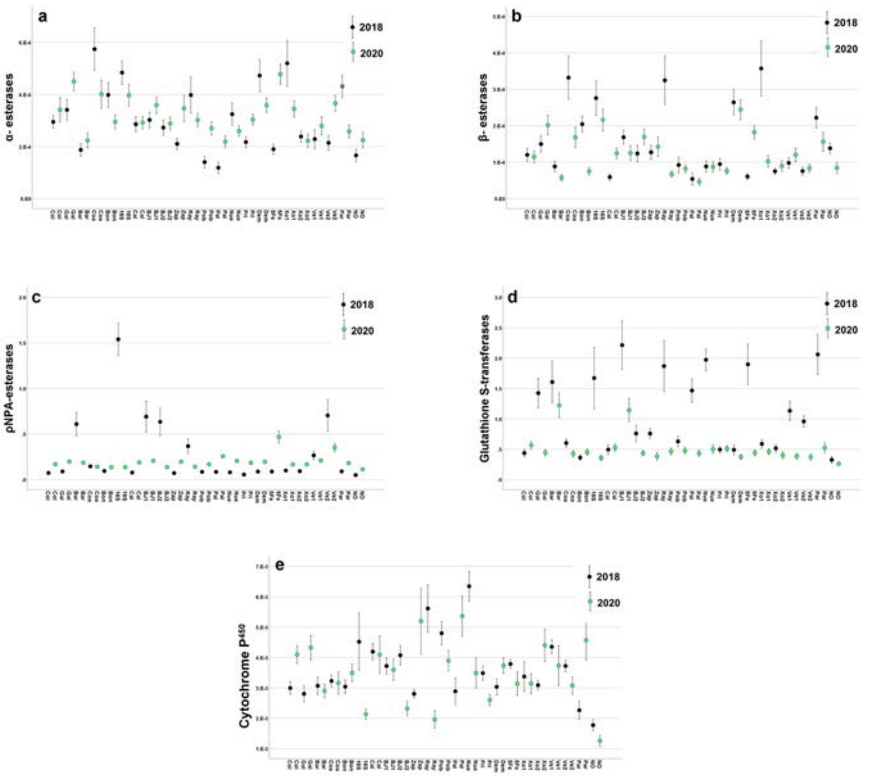

FIGURE 2. MEANS AND STANDARD DEVIATIONS (±SD) OF ENZYME ACTIVITY AMONG *AE. AEGYPTI* POPULATIONS COLLECTED FROM 22 SITES IN TAPACHULA, CHIAPAS, MEXICO IN 2018 AND 2020: (A) α-ESTERASES, (B) β-ESTERASES, (C) ρNPA-ESTERASES, (D) GLUTATHIONE S-TRANSFERASAS, (E) CYTOCHROMES P^{450}

Enzyme levels between 2018 and 2020

Only Gal mosquitoes showed an increase in the levels of α- and β-esterases, ρNPA-esterases, and cytochromes P^{450} from 2018 to 2020 ($p<0.01$, tables I and II), whereas their GST levels declined significantly ($p<0.0001$). The α-esterases levels of Gal, Bj1, Zap, Pob, Pal, Pri, 5Fe, and Ve2 mosquitoes increased significantly ($p<0.01$). β-esterases increased significantly in Gal, Cal, Bj2 and 5Fe ($p<0.01$). The mosquitoes of 5Fe had the greatest levels of α- and β-esterases ($p<0.0001$). The GST activities of Col and Bon increased significantly ($p<0.01$). The activity of ρNPA-esterases increased significantly in Col, Gal, Bon, Cal, Zap, Pob, Pal, Nue, Pri, Dem, 5Fe, Xo1, Xo2 and Par ($p<0.0001$), with Pri showing the greatest rise.

Significantly elevated amount of cytochromes P^{450} were detected in mosquitoes from Col, Gal, Zap, Pal, Dem, Xo2 and Par ($p<0.02$), with those of Gal with the highest levels ($p<0.0001$) in 2020. The enzyme levels of the remaining mosquito populations fell between 2018 and 2020.

From 2018 to 2020, the levels of β- esterasas, ρNPA-esterases and GST among Tapachula´s sites decreased significantly ($p<0.001$, tables I and II). The levels of α-esterases and cytochromes P^{450} did not differ over the course of two years in relation to N.O. levels.

Correlations between enzyme levels and insecticide resistance ratios[17]

The correlation between previously published Malathion RRs and AChEi (r=0.459) was statistically significant ($p=0.03$), but not for other enzymes. The correlations between the RRs of Clorpirifos, Bendiocarb, Deltamethrin and Permethrin and the levels of the six enzymes were not statistically significant ($p>0.05$).

Bioassay with synergists of PYR

Mean and Standard deviation mortality percentages were: for Permethrin 15±5.54, for Permethrin/PBO 65.7±10.68, for Permethrin/DEF 80.3±13.06, for Permethrin/DM 50.45±20.42.

Discussion

The initial response of insects to insecticide exposure is an increase in their detoxification activity.[24] In general, esterases are involved with the metabolism of OP;[25,26] however, they also contribute to cross-resistance with PYR and CARB.[10] GSTs confer resistance through either direct metabolism of some insecticides or indirect protection against oxidative stress caused by insecticide exposure.[27,28] In Mexican populations of *Ae. aegypti*, resistance to PYR has been attributed to elevated levels of GST,[9] especially in mosquito populations resistant to permethrin and deltamethrin from Tapachula in 2018.[17] In this study, we report high levels of GST in 11 of 22 sites in 2018, confirming that it is a support mechanism in the oxidative stress caused by insecticide exposure. This latter mechanism likely favors deltamethrin resistance more than permethrin resistance, as permethrin resistance is particularly associated with *kdr*.[29] We also reported allele frequencies ranging from 0.16 to 0.7 for V1016I and from 0.85 to 1 for F1534C in the same 2018 populations.[17] Since 2010, vector control programs in Mexico have decreased the use of PYR, however, commercial insecticides are commonly sprayed in households inside and outside houses, so mosquitoes are likely being selected for its main component, the PYRs.

In Tapachula, we found 15 of 22 sites exhibiting high levels of α-esterases. Therefore, it cannot be ruled out that elevated α-esterases are related with tolerance to pyrethroids in Mexico.[8] In our previous study[17] we identified moderate chlorpyrifos resistance in 13 sites (RR= 5.2-7.2) and high chlorpyrifos resistance in Pal (RR = 10). From these, nine sites had significantly elevated levels of α-esterases, suggesting they likely contribute to chlorpyrifos resistance. *Aedes aegypti* populations from Cuba, Costa Rica,[30] and Veracruz, Mexico[31] were found having high levels of esterases and resistance to chlorpyrifos. In Mexico, chlorpyrifos is permitted for use in vector control, and it has been deployed in Tapachula in recent years as an ultra-low-volume space spray (ULV).[32] High ρNPA- esterase activity was also detected in 21 sites collected from Tapachula in both years. In addition 20 of these mosquito populations also had high levels of cytochromes P^{450}, which rose by 2020.

Cytochromes P^{450} have been identified in *Ae. aegypti* PYR-resistant populations from Selangor, Malaysia.[33] These enzymes are involved in detoxification, as confirmed by the use of PBO synergizing with permethrin[34] However, they are also involved in the bioactivation of OPs, converting them from their phosphorothioate form to a toxic oxon form that inhibits AChE.[35] AChE is the primary target of OPs and CARB; hence it becomes insensitive when mutations are selected under the persistent use of these insecticides.[36] Although no mutation has been identified in this enzyme in the dengue vector, we were able to identify propoxur-resistant mosquitoes among Tapachula sites, ranging from 2 to 52 out of 97 insects analyzed per site.

The elevated enzyme levels found in *Ae. aegypti* populations from Tapachula are strong indicators of their involvement in metabolic resistance to deltamethrin, permethrin, and chlorpyrifos.[17] The combination of enzymes is typically more effective than the increase of a single family of enzymes in particular.[11] The results using synergists, confirmed that resistance to PYR is also based on its metabolism, where the three groups of enzymes help detoxifying. Esterases contributed more, since when they were inhibited by DEF, mortality increased by 65%, followed by Cytochromes P^{450} (PBO) and GST (DM), whose inhibition increased mortality by 50 and 35%, respectively.

Enzyme levels in Tapachula mosquitoes were maintained high from 2018 to 2020 at most sites, decreasing esterases at a few, but increasing GSTs at many. If there was a correlation with dengue cases in Tapachula, we cannot elucidate it, since the decrease from 2018 (504) to 2020 (27), was more due to the confinement of people due to the Covid-19 pandemic.

These results demonstrate the value of incorporating biochemical assays into resistance monitoring as part of vector control programs activities, in order to obtain a diagnosis of the mechanisms of resistance and thereby improve the management strategies for insecticide resistance in this vector.

Declaration of conflict of interests. The authors declare that they have no conflict of interests.

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