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Comparison of Insecticide Resistance and Its Enzyme Mechanisms among *Aedes aegypti* Collected with Three Methods in a Dengue-Endemic City in Southern Mexico

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Abstract

Background: Despite the physical and chemical effort to control *Aedes aegypti*, the arboviruses transmission in the south of Mexico remains latent. Trying to improve the methods of entomological surveillance routinely used, whether the estimation of resistance to insecticides used for its control, as well as their enzyme mechanisms, were influenced by the phase in which the mosquitoes were collected through three different collection methods was investigated. **Materials and Methods:** Mosquito collections from the "5 de Febrero" neighborhood in Tapachula, Mexico were obtained by ovitraps, larvitraps, and a CDC backpack aspirator. Insecticide resistance of F_1 females was determined by WHO diagnostic doses and resistance ratios (RR_{50}), furthermore, levels of insecticide metabolism enzymes were determined by biochemical assays. **Results:** Overall, in mosquitoes collected by ovitraps, larvitraps, and CDC backpack aspirator respectively, the low mortalities obtained with the discriminant dose to Malathion (27.57%, 26.97%, and 26.91%), and to Bendiocarb (50.5%, 45.36%, and 54.97%) suggest resistance. However, LC_{50} for Malathion (0.922, 0.934, and 0.915) and for Bendiocarb (0.112, 0.109, and 0.107); and the low resistance ratios (RR_{50}) for Malathion (3.34, 3.29, and 3.27) and for Bendiocarb (2.15, 2.1, and 2.06) does not suggest resistance. Although a slight numerical variation is observed between the three LC_{50}

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values, the overlap observed between their confidence intervals allows us to assume that there were no differences between the three methods. In general, esterases (determined with three substrates), glutathion S-transferases (GST) and cytochromes P^{450} were statistically higher than those of the susceptible strain; and the three enzyme levels were statistically different among the three collection methods ($P < 0.01$), being those collected with CDC backpack aspirator with the highest levels. **Conclusion:** Although using a CDC backpack aspirator demonstrated being the best collection method determining a specific resistance mechanism (as elevation at the enzyme level) in the mosquito adult phase, any collection method is reliable to determine whether a field mosquito population is resistant or susceptible to an insecticide.

Keywords

CDC Backpack Aspirator, Insecticide Resistance, Larvitraps, Mosquitoes, Ovitraps

1. Introduction

Aedes aegypti, is the main vector of dengue, chikungunya and Zika virus in America, this mosquito is distributed in the tropical and subtropical regions of America, South East Asia and Africa. The permanence in these areas has been influenced by improvised urbanization trends and climate variability [1]. Dickens *et al.* (2018) [2] suggest that human accessibility and biological plasticity of these mosquitoes are critical parameters for their distribution.

The initiative to control this vector in the Americas began in the early 20th century in 1901 by William C. Gorgas, with the elimination of containers considered potential breeding sites for mosquitoes that transmit yellow fever [3]. It is known that by 1945 the insecticide DDT was first used in South America for the eradication of *Ae. aegypti* in Bolivia [3]; in 1947 the proposal to eradicate *Ae. aegypti* is accepted and promoted by all PAHO members [4]. By 1967, the campaigns had yielded positive results in 18 countries, confirming the eradication of *Ae. aegypti.* However, between the 1970s and 1990s the re-emergence of *Ae. aegypti* was evidenced by the emergence of multiple dengue outbreaks in different countries of South, Central and North America; this was due to the deterioration of control programs [5]. Brady *et al.* (2012) [6] mentioned that approximately 390 million cases of dengue virus infection occur annually in tropical and subtropical areas around the world, where 824 million people in 128 countries are at risk of infection from living in urban areas. This is why in 2021, the transmission of dengue, chikungunya and Zika is still present in the region of the Americas [7].

Currently, a vaccine "CYD-TDV or Dengvaxia" (Sanofi Pasteur) is designed but not approved for dengue in all countries. It has presented clinical disadvantages, increasing the risk of severe dengue infection in children aged 2 to 5 years

and in people infected for the first time after vaccination [8] [9]. That is why Public Health systems continue to bet on vector control programs, focused on the elimination of *Ae. aegypti* populations through integrated management systems.

The presence of *Ae. aegypti* in urbanized areas is closely associated with the human habitat, this mosquito lays its eggs in artificial hatcheries close to houses, such as: cisterns, tanks, pools, plastic buckets, tires and pots; inadequate management or accumulation of these makes them potential breeding sites, thus contributing to the abundance of these mosquitoes [10].

In Mexico, actions for the control of *Ae. aegypti* focuses on the elimination of mosquito breeding sites with the use of larvicides, residual and spatial sprays with adulticides, and with health promotion [11]. Chemical control is subject to high quality standards in the selection, approval and use of insecticides by the National Center for Preventive Programs and Disease Control (CENAPRECE), ensuring the effectiveness of the products as well as of the application techniques by means of biological efficacy tests [12].

However, in recent years, chemical control of *Ae. aegypti* is threatened by the emergence of mechanisms of resistance to the main groups of insecticides: carbamates, organophosphates and pyrethroids in the Americas, Asia and Africa [13]. Bisset (2002) [14] suggests that prolonged use, misdosing and inappropriate application of insecticides within these groups has resulted in the selection of one or more resistance mechanisms in mosquitoes, such as mutations at the target site and increased detoxification of insecticides in mosquitoes, regulated primarily by the activity of certain enzymes such as esterases, glutathione S-transferases and cytochromes P^{450}.

The World Health Organization (WHO) and the Center for Disease Control and Prevention (CDC) have established first-line procedures and tools for monitoring insecticide susceptibility in mosquitoes with the use of "tube kit with impregnated paper" [15] and "CDC bottles" [16], as well as biochemical and molecular analyses for the characterization of the enzyme based- and mutations based-mechanisms.

The diversity of collection methods for the colonization of mosquitoes used in tests of susceptibility to insecticides is wide, obtaining biological material in different biological phases: eggs, larvae/pupae and imagos of *Ae. aegypti*. It seems that the choice of collection method so far has been for its practicality, abundance of collected mosquitoes, time and effort.

Currently in Mexico *Ae. aegypti* is collected in phase of egg with the use of ovitraps [17], reliable and cost-effective method; other authors who have monitored insecticide resistance in some South American countries such as Ecuador and Colombia obtain their wild strains through larval surveys [18] [19], as recommended by WHO in its *Ae aegypti* surveillance procedure [20]. The capture of imagoes of field *Ae. aegypti* has been another option in Venezuela for the colonization of mosquito strains for the estimation of resistance to insecticides [21]. Other authors prefer not to mention the capture technique of field strains

of mosquitoes used in estimating resistance to insecticides in Peru [22].

However, it is still unknown whether the physiological stage in which *Ae. aegypti* is collected to be colonized for these studies influences the estimation of insecticide resistance levels. If so, a sub-or overestimation of the resistance due to the phase in which it is collected could have important implications for the effectiveness of monitoring it and therefore for its control.

Trying to improve the methods of entomological surveillance routinely used by the vector control program, the resistance levels to Malathion and Bendiocarb (two insecticides widely used today in Mexico), and its enzyme mechanisms of *Ae. aegypti* collected in different stages of life using the ovitrap, larvitrap, and CDC backpack aspirator methods, were evaluated and compared for differences.

2. Materials and Methods

2.1. Study Site

Ae. aegypti mosquitoes were collected in the "5 de Febrero" neighborhood located northeast of Tapachula, Chiapas, N14˚55'09.120"W 92˚15'32.82"W (**Figure 1**), at 160 meters above the sea level, average annual temperature from 24˚C to 35˚C, and rainfall ranging from 2300 to more than 3900 mm per year. This neighborhood has been frequently selected for studies of insecticide resistance

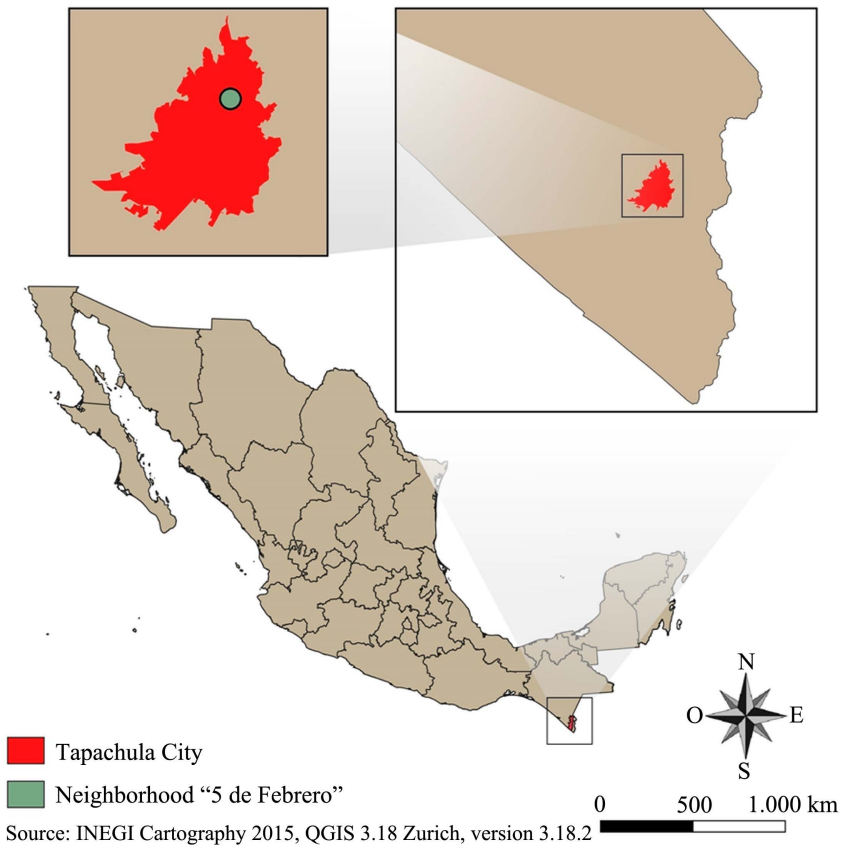

Figure 1. Study site map. Geographical location of the "5 de Febrero" neighborhood where the collections of *Ae. aegypti* in the field.

[23] [24], due to the abundant presence and distribution of some species of culicids in the urban area and areas of undisturbed vegetation, and the constant use of adulticides.

2.2. Collection and Mosquito Breeding Sites

Ae. aegypti eggs, larvae/pupae and imagos were collected during five consecutive weeks, using three collection methods: ovitraps, larvitraps and CDC backpack aspirator, respectively. The ovitraps consisted of 1-litre black plastic canisters fitted with filter paper and were used as indicated in the national operational guide [17]. Likewise, modified larvitraps with the capacity to contain 3 liters of water were used for the collection of larvae and pupae of *Ae. aegypti* [25]. On the other hand, for the collection of imagos of *Ae. aegypti* intra- and peri-domiciliary aspiration were used with CDC backpack aspirator approximately between 15 and 20 minutes as indicated by the entomological collection guide of the INDRE [26]. The collection was carried out intra and peri domiciliary every 7 days with the due informed consent of the community. The biological material collected was transferred to the insectarium of the Insecticide Resistance Laboratory at the Centro Regional de Investigación en Salud Pública (CRISP) to obtain the F_1 mosquito generation under 27˚C - 30˚C, 70% relative humidity, and 12:12 (light: dark) photoperiods. The methodological guide for the installation and maintenance of insectarium of *Ae. aegypti* (Diptera: culicidae) [27] was used for this purpose.

2.3. Susceptibility Bioassays

The susceptibility studies were undertaken according to the WHO methodology [15] [28]. Whatman #1 filter papers were manually impregnated with the diagnostic doses 0.8% of Malathion (technical grade, 98.5% purity), and 0.1% of Bendiocarb (100% purity), both from Sigma Aldrich. To determine the lethal concentration at 50% (LC_{50}) the following concentrations were used for field mosquitoes: Malathion 1.6%, 1.3%, 1.0%, 1.8%, 0.5%, and for Bendiocarb 0.2%, 0.15%, 0.1%, 0.08%, 0.05%. While for the susceptible New Orleans mosquitoes were used: for Malathion 0.8%, 0.5%, 0.3%, 0.1%, 0.07% and for Bendiocarb 0.1%, 0.08%, 0.05%, 0.03%, 0.01% (**Table 1**). Sugar fed female mosquitoes 2-3 day old were exposed to the insecticide and after 1 h mosquitoes were passed to the resting tubes during 24 h, then mortality readings were registered. Three replicates with four tubes each were performed for each mosquito population and each insecticide concentration (**Table 1**). All evaluations were carried out with its respective control tube using olive oil impregnated paper. The WHO criterion was used for susceptibility/resistance diagnosis of mosquito populations: susceptible from 98% to 100% mortality, resistance to be confirmed from 90% to 98% mortality, and resistant mosquitoes < 90% mortality. As to interpret the resistance ratios (RR_{50}): susceptibility (5×), moderately resistant (5× to 10×) and resistant (10×) [15] [29].

Table 1. Insecticides, concentrations and number of mosquitoes used to determine resistance levels in *Ae. aegypti* from three collection methods in Tapachula, Chiapas.

Insecticide	Group	Strain	n^1	DC%[2]	n^3	Concentration Scale %
Malathion (Sigma Alcdrich)	Organophosphate	"5 de Febrero" (three collection methods)	906	0.8	4531	1.6, 1.3, 1.0, 1.8, 0.5
		New Orleans (Susceptible)	300		1506	0.8, 0.5, 0.3, 0.1%, 0.07
Bendiocarb (Sigma Aldrich)	Carbamate	"5 de Febrero" (three collection methods)	905	0.1	4516	0.2, 0.15, 0.1, 0.08, 0.05
		New Orleans (Susceptible)	301		1508	0.1, 0.08, 0.05, 0.03, 0.01

1. Number of mosquitoes exposed to diagnostic concentration. 2. Diagnostic concentration in percentage (%). 3. Number of mosquitoes exposed to Concentration scale (%).

2.4. Biochemical Tests

The levels of esterases using α- and β-naphtyl acetate, and ρ-nitro phenyl acetate (ρNPA) as substrates, glutathione S-transferases (GST) and cytochromes P^{450} were performed following the protocol described by Penilla and cols. [30] in *Ae. aegypti* collected with ovitraps, larvitraps and CDC backpack aspirator. All enzyme levels of the field mosquitoes were compared with that obtained in a laboratory susceptible strain New Orleans.

2.5. Statistical Analysis

Malathion and Bendiocarb LC_{50} for field mosquito populations were obtained through the cumulative probability analysis under its curve (Probit), analyzing mortality rates in the R 3.5 statistical package. The enzyme levels of field mosquitoes and those of the susceptible strain were compared using a variance analysis (ANOVA) with a Post Hoc to find variability between the mosquito strains and the used collection methods. Statistical analysis and histograms were performed with the IBM SPSS Statistic 21.0.

3. Results

3.1. Bioassays of Susceptibility

Field mosquito mortalities to the diagnostic concentration of Malathion and Bendiocarb were lower than the 100% mortality of the susceptible strain. Mortalities for ovitraps, larvitraps and CDC backpack aspirator respectively were 27.57%, 26.97%, and 26.91% for Malathion; and 50.5%, 45.36%, and 54.97% for Bendiocarb (**Figure 2**), suggesting resistance.

LC_{50} for ovitraps, larvitraps and CDC backpack aspirator respectively were 0.922, 0.934, and 0.915 for Malathion; and 0.112, 0.109, and 0.107 for Bendiocarb (**Figure 3**). The RR_{50} or number of times that LC_{50} from field mosquitoes were greater than LC_{50} from the susceptible strain ranged from 1.91 to 2.23 for Malathion and 2.02 to 2.18 for Bendiocarb (**Table 2** and **Table 3**), suggesting susceptibility for both insecticides. It should be noted that, given the nature of the data,

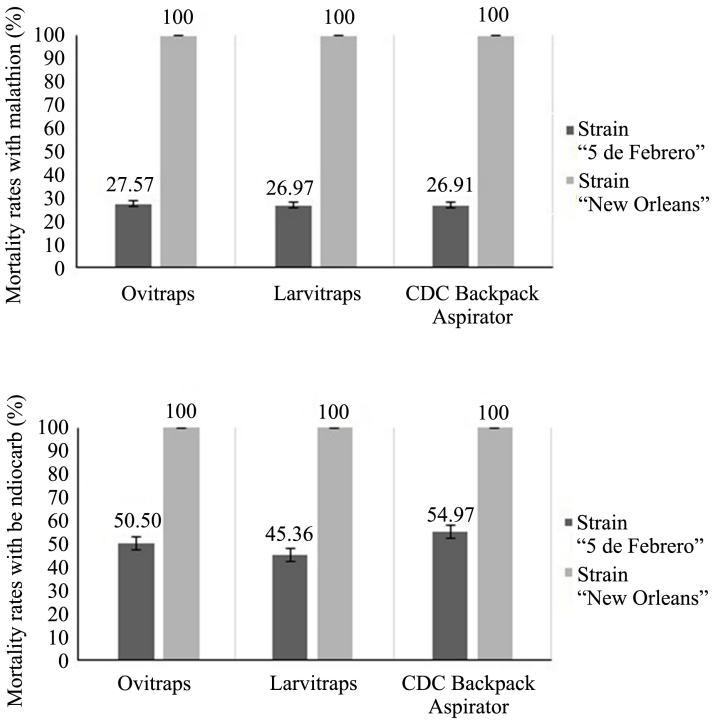

Figure 2. Above: Mortality rates of *Ae. aegypti* "5 de Febrero" and New Orleans exposed to insecticide malathion and; below: mortality rates of *Ae. aegypti* "5 de Febrero" and New Orleans exposed to the insecticide bendiocarb.

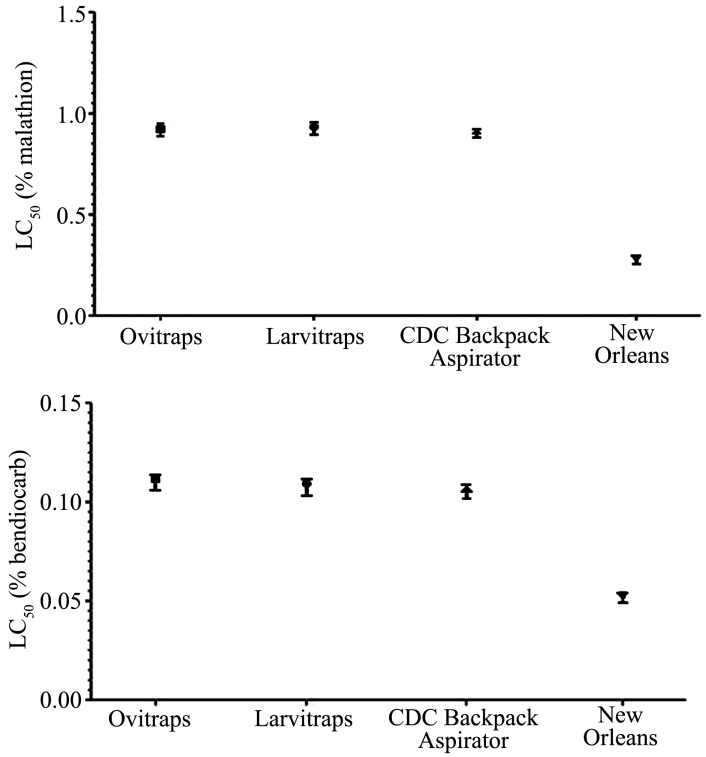

Figure 3. Above: Lethal concentration fifty of *Ae. aegypti* "5 de Febrero" and New Orleans exposed to insecticide malathion and; below: lethal concentration fifty of *Ae. aegypti* "5 de Febrero" and New Orleans exposed to insecticide bendiocarb.

Table 2. Resistance status of *Aedes aegypti* adults to insecticide malathion, compared to the reference New Orleans strain.[1]

Strain by collection method	n^2	LC_{50} (95% CI)	LC_{99} (95% CI)	Slope ± SE	Ji 2	P value	RR_{50}	RR_{99}
"5 de Febrero" (Ovitraps)	1506	0.922 (0.891 - 0.932)	1.458 (1.380 - 1.483)	4.472 ± 0.209	1085.50	<2.2e−16	3.29	1.92
"5 de Febrero" (Larvitraps)	1519	0.934 (0.896 - 0.946)	1.690 (1.332 - 1.509)	3.166 ± 0.140	813.3	<2.2e−16	3.34	2.23
"5 de Febrero" (CDC Backpack Aspirator)	1506	0.915 (0.884 - 0.925)	1.452 (1.374 - 1.477)	4.462 ± 0.209	1081.20	<2.2e−16	3.27	1.91
New Orleans (Susceptible)	1506	0.280 (0.256 - 0.288)	0.759 (0.688 - 0.782)	5.019 ± 0.232	848.84	<2.2e−16	1	1

1. LC_{50} and LC_{99}, lethal concentration that affects 50% and 99% of the population, respectively; RR_{50}, resistance ratio calculated as the ratio between the LC_{50} of field mosquitoes and the susceptible strain, as well as for RR_{99}. 2. Number of mosquitoes exposed.

Table 3. Resistance status of *Aedes aegypti* adults to insecticide bendiocarb, compared to the reference New Orleans strain.[1]

Strain by collection method	n^2	LC_{50} (95% CI)	LC_{99} (95% CI)	Slope ± SE	Xchi2	P value	RR_{50}	RR_{99}
"5 de Febrero" (Ovitraps)	1504	0.112 (0.106 - 0.114)	0.246 (0.227 - 0.252)	17.931± 0.834	604.93	<2.2e−16	2.15	2.18
"5 de Febrero" (Larvitraps)	1506	0.109 (0.103 - 0.111)	0.240 (0.221 - 0.246)	18.389 ± 0.856	614	<2.2e−16	2.1	2.12
"5 de Febrero" (CDC Backpack Aspirator)	1506	0.107 (0.101 - 0.109)	0.227 (0.210 - 0.233)	20.020± 0.918	676.61	<2.2e−16	2.06	2.02
New Orleans (Susceptible)	1508	0.052 (0.049 - 0.053)	0.113 (0.105 - 0.115)	39.700 ± 1.613	932.28	<2.2e−16	1	1

1. LC_{50} and LC_{99}, lethal concentration that affects 50% and 99% of the population, respectively; RR_{50}, resistance ratio calculated as the ratio between the LC_{50} of field mosquitoes and the susceptible strain, as well as for RR_{99}. 2. Number of mosquitoes exposed.

they were not statistically analyzed to determine possible differences between the LC_{50} values obtained between the collection methods for each insecticide. Therefore, although a slight numerical variation is observed between the three LC50 values (see **Figure 3**), the overlap observed between their confidence intervals allows us to assume that there were no differences between the three methods.

3.2. Biochemical Tests

***α-β-* and *ρ*NPA-esterases:** The three enzyme levels from the mosquito population were significantly different ($P < 0.01$) between collection methods, except for *β*-esterases in larvitraps vs ovitraps, and for *α*-esterases and *ρ*NPA-esterases in CDC backpack aspirator vs larvitraps. In general, the three enzyme levels were statistically higher when compared with the susceptible strain levels (**Figure 4**). But when separated by collection method and type of esterases, mosquitoes collected with ovitraps and larvitraps were not significantly different in their *ρ*NPA-esterase levels when compared to those of the susceptible strain. Concentration ratios (CR) of *α-β-* and *ρ*NPA-esterases higher than those of the susceptible

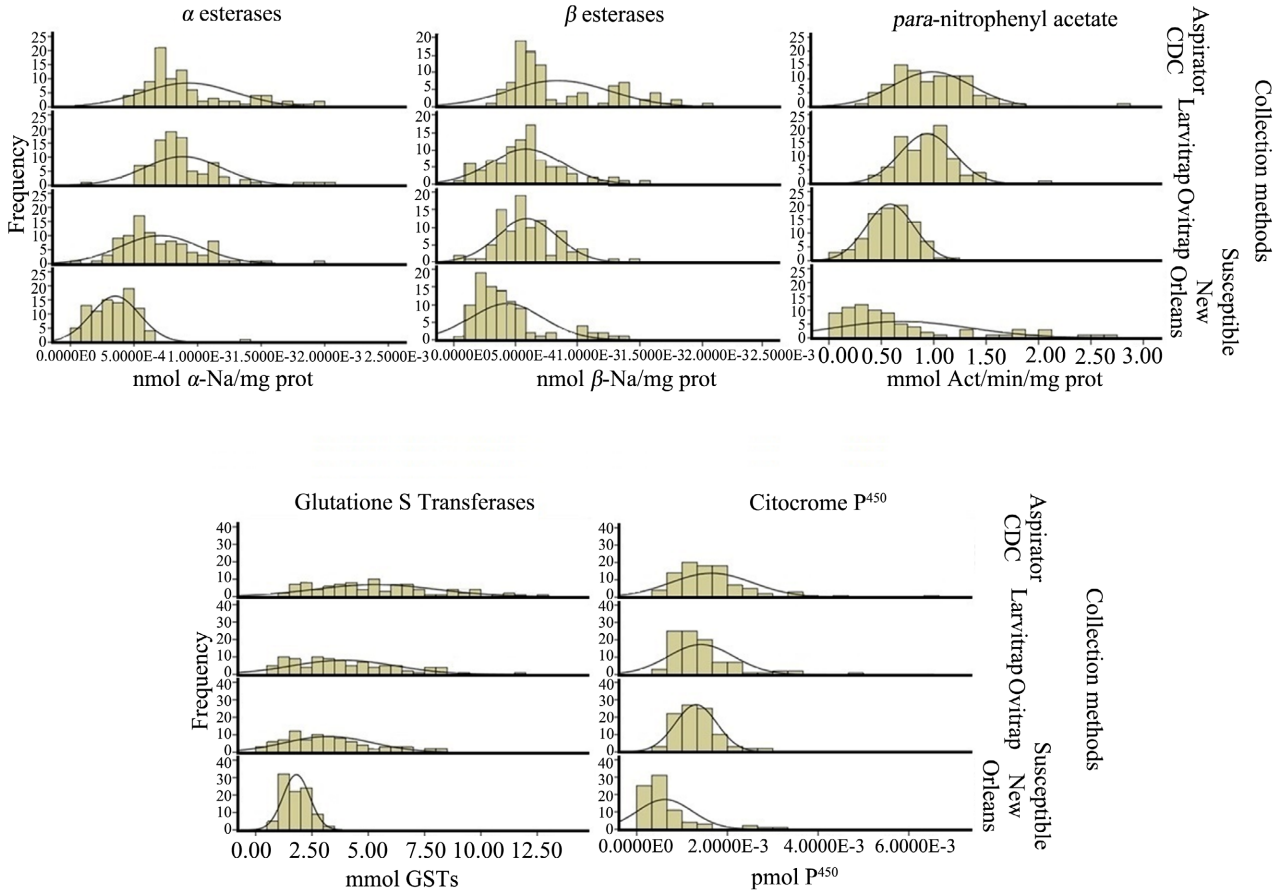

Figure 4. Concentration of esterases with three substrates (*α*, *β* naphthyl acetate and ρNPA), GST activity and Cytochrome P^{450} content in *Ae. aegypti* collected in the field, against the susceptible strain New Orleans.

strain were 2.0, 1.4 and 0.8 times for ovitraps; 2.51, 1.4, 1.4 times for larvitraps; and 2.63, 1.98 and 1.43 times for CDC backpack aspirator (Table 4). **Glutathione S-transferase:** GST levels of field mosquitoes were significantly different ($P < 0.01$) among collection methods, and against the susceptible strain (Figure 4). Mosquitoes from ovitraps, larvitraps, and CDC backpack aspirator had CR: 1.8, 2.2, and 2.9 times respectively higher than the susceptible strain (Table 4), with those collected with CDC backpack with statistically differences ($P < 0.01$). **Cytochrome P^{450}:** field mosquito levels collected with ovitraps, larvitraps, and CDC backpack had CR of 2.12, 2.29, and 2.67, respectively higher than the susceptible strain (Figure 4 and Table 4). With statistical differences only between mosquitoes collected with CDC backpack aspirator vs ovitrap, and CDC backpack vs the susceptible strain ($P < 0.01$).

4. Discussion

The management of resistance to insecticides is an important component in any vector control program [31], since it depends on this to identify that a possible failure in mosquito control is due to the insecticide in use [32] and, therefore, the recommendation for the alternative insecticide can be made based on the

Table 4. Mean of the enzymatic activity of *Aedes aegypti* collected with three collection methods, represented in number of times greater (CR) than the enzymatic activity of the susceptible strain New Orleans.

Collection method	α naftil acetato (nmol/mg prot)	±SE	CR[1]	β naftil acetato (nmol/mg prot)	±SE	CR[1]	ρNPA[2] (mmol Act/min/mg prot)	±SE	CR[1]	GST[3] (mmol)	±SE	CR[1]	Cytochrome P^{450} (pmol)	±SE	CR[1]
Ovitraps	0.00070	0.000032	2.0	0.00059	0.000026	1.4	0.58	0.024	0.8	3.23	0.21	1.8	0.0013	0.0013	2.1
Larvitraps	0.00088	0.000032	2.5	0.00058	0.000031	1.4	0.94	0.027	1.4	3.90	0.24	2.2	0.0014	0.0014	2.3
CDC Backpack Aspirator	0.00093	0.000038	2.6	0.00084	0.000043	2.0	0.99	0.039	1.4	5.31	0.28	2.9	0.0016	0.0001	2.6
New Orleans	0.00035	0.000020	1.0	0.00043	0.000031	1.0	0.69	0.075	1.0	1.81	0.06	1.0	0.0006	0.0001	1.0

1. Concentration ratio (CR). 2. *para*-nitrophenyl acetate. 3. Glutation S-transferase.

evidence. However, for the implementation of an insecticide resistance monitoring system, it is also necessary to have an entomological surveillance system, whose objective is not only to measure changes in the vector population, but also to provide viable and abundant biological material for the laboratory studies. Here, the resistance to Malathion and Bendiocarb were determined in a population of *Ae. aegypti* from a neighborhood with a high story of insecticide usage, and whether the type of collection method used influenced somehow with the levels of insecticide resistance estimated was investigated. The WHO recommends the use of biological material collected by larval surveys for biological testing in the monitoring of insecticide resistance [20], because this method provides a greater number of specimens for colonization, but with greater effort, number of staff and extensive collection coverage. In other studies, the material is collected by ovitraps [22] [23] [33] [34], one of the more versatile and reliable collection method known. However, we should not neglect the economic investment needed for its manufacture and the use of filter paper or pellon fabric, and some more tools mentioned in the methodological guide for entomological surveillance with ovitraps (CENAPRECE) [17]. On the other hand, the collection of mosquitoes with the use of equipment such as BG-Sentinel or CDC backpack aspirator becomes a much more intrusive method than the previous ones, requiring more investment of time, equipment and trained personnel, which indicates greater economic investment [35].

As mentioned above, the WHO recommends, but does not impose the method of collection, nor the stage at which mosquitoes should be collected for the biological testing. Therefore, the implementation of another collection method such as the use of larvitraps (method to collect larvae and pupae of mosquitoes), can be another option and in turn can serve to systematize the larval research. Moreover, when this collection method has proved to be effective [36] even significantly more effective in the collection of *Ae. aegypti* compared to the use of

ovitraps [25] [37].

No evaluations of comparing insecticide resistance in a population of *Ae. aegypti* mosquitoes from different collection methods has been reported. The results of the present study show that resistance obtained by LC_{50} of mosquitoes exposed to Malathion and Bendiocarb were not different among the collection methods. Contrary to the biochemical assays results, where the highest levels of most of the enzymes were recorded in mosquitoes collected with CDC backpack aspirator, with statistical differences for β-esterases and GST ($P < 0.01$). The high levels of α- and β-esterases, GSTs, and cytochromes P^{450} found in mosquitoes from larvitraps, ovitraps, and CDC backpack aspirator compared to those observed in the susceptible strain evidence the relationship with Malathion and Bendiocarb resistance obtained with the WHO diagnostic doses. High levels of esterases are involved with resistance to organophosphates, carbamates and pyrethroids [38] [39], corroborating that the biochemical assays are more sensitive in detecting variations at the enzyme level [30] vs the results obtained by WHO tube bioassays. On the other hand, in Mexico, insecticide resistance in *Ae. aegypti* has been widely described for different populations [23] [24] [33] [38] of this vector, a situation that has been reported as a serious problem for its control measures and strategies [32]. There are already records of the resistance to organophosphates and carbamates in mosquitoes collected using ovitraps and evaluated for insecticide resistance with the CDC method. López *et al.* (2016) [23] reported mortalities minor of 80% for Malathion and between 88% and 91% for Bendiocarb. Our results confirm the resistance to Malathion and Bendiocarb in *Ae. aegypti* adults diagnosed with the WHO diagnostic dose with the WHO tubes. This suggests that regardless of the type of bioassay, when using both CDC and WHO diagnostic concentrations, the findings of resistance levels are similar. Moreover, when calculating LC_{50} to obtain the RR, there was no resistance. The susceptible strain used in this study may be in a lower range of susceptibility than the susceptible strain or strains of *Ae. aegypti* used for the calculation of the diagnostic concentration by the WHO. However, calculating lethal concentrations with the CDC method and with these same insecticides, we have found resistance ratios in the same ranges, comparing the same susceptible and field strains.

5. Conclusion

Resistance levels in *Ae. aegypti* from "5 de Febrero", were higher compared to the susceptible strain New Orleans using both the WHO diagnostic concentration and the RR_{50}, but only results from the former method determined the mosquito population as resistant. Insecticide resistance levels to Malathion and Bendiocarb were not different between collection methods. While differences in levels of esterases, GSTs and cytochromes P^{450} were statistically significant among mosquitoes from different collection methods, and most were also higher compared to the levels of the susceptible strain, indicating that more than one resis-

tance mechanism based on the metabolism of the insecticides is involved. These could be explaining the low mortalities found using the WHO diagnostic doses, so enzymes could play an important role in the resistance to Malathion and Bendiocarb in this mosquito population; however metabolism studies are required for confirmation. We are now certain that the collection method used to obtain F_1 generation mosquito colonies for insecticide susceptibility bioassays does not influence the results, and choosing any of them rather depends on the different situations of economy, logistics, operating personnel and objectives of those interested in carrying out these studies.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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