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Original article

Determination of insecticide resistance-associated insensitive acetylcholin-esterase in *Culex quinquefasciatus* Say, 1823 (Diptera: Culicidae) collected in Yucatán, Mexico

Determinación de la acetilcolinesterasa insensible asociada a la resistencia a insecticidas en *Culex quinquefasciatus* Say, 1823 (Diptera: Culicidae) colectados en Yucatán, México

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ABSTRACT. Acetylcholinesterase is an enzyme whose main function is to terminate the transmission of nerve impulses in the cholinergic synapse in insects through the hydrolysis of acetylcholine. However, frequent applications of insecticides can select for individuals with an alteration in the enzyme and functional changes that result in an insensitivity to insecticides. In Mexico, the current strategy to combat insect vectors such as *Culex quinquefasciatus* is based mainly

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on the use of synthetic insecticides. The use of these chemicals in health and agricultural practices may also contribute to mosquito insensitivity to several groups of insecticides. Therefore, it is considered necessary to determine if there is any physiological mechanism associated with resistance. For this reason, the objective of this research was to identify if there is insensitivity of acetylcholinesterase to insecticides in *Cx. quinquefasciatus* populations, collected in urban and rural areas of the State of Yucatán, Mexico. Enzymatic tests were carried out with slight modifications to the established protocols. Areas were identified with mosquito populations showing acetylcholinesterase insensitivity to propoxur, a carbamate insecticide. We consider it important to determine the environmental factors that affect the behavior and response of mosquitoes following exposure to insecticides, and this knowledge could inform the design of efficient strategies in vector control programs.

Key words: mosquitoes; biochemical tests; enzymatic insensitivity; surveys; rural; urban; areas

RESUMEN. La acetilcolinesterasa es una enzima, cuya principal función es finalizar la transmisión de los impulsos nerviosos en la sinapsis colinérgica en los insectos mediante la hidrólisis de la acetilcolina. Sin embargo, el uso frecuente de los insecticidas sintéticos, probablemente originen una alteración en la enzima y cambios funcionales conduciendo a la insensibilidad a los insecticidas neurotóxicos. En México la estrategia actual para el combate de insectos vectores como *Culex quinquefasciatus*, se basa principalmente en el uso de estos compuestos. El uso de estos productos químicos en áreas de la salud y prácticas agrícolas probablemente contribuye a la insensibilidad a diferentes grupos de insecticidas. Por lo tanto, se considera necesario determinar si existe algún mecanismo fisiológico asociado a la resistencia. Por tal motivo, el objetivo de esta investigación fue identificar si existe insensibilidad de la acetilcolinesterasa a insecticidas en poblaciones de *Cx. quinquefasciatus*, colectados en áreas urbanas y rurales en Yucatán. Se realizaron pruebas enzimáticas con ligeras modificaciones en los protocolos establecidos y se identificaron áreas con poblaciones de mosquitos con acetilcolinesterasa insensible a propoxur, que es un carbamato ampliamente utilizado en México. Con nuestro trabajo consideramos importante identificar los factores ambientales que afectan el comportamiento y respuesta de los mosquitos a los insecticidas, lo cual será de gran utilidad para el diseño eficiente de estrategias en los programas de control de vectores.

Palabras clave: mosquitos; pruebas bioquímicas; insensibilidad enzimática; encuestas; zonas rurales; zonas urbanas

INTRODUCTION

Acetylcholinesterase (AChE) is an enzyme found in insects whose main role is to terminate the transmission of nerve impulses in the cholinergic synapse via the rapid hydrolysis of acetylcholine (Zhao *et al*., 2014). The inhibitory activity of organophosphate and carbamate insecticides leads to functional modifications in AChE receptors, resulting in the inhibition of nerve signal transmission (Lopez, 2010). The frequent application of organophosphate and carbamate insecticides can select for individuals with altered AchE that is insensitive to the active ingredients of neurotoxic insecticides (WHO, 2006). Acetylcholinesterases are enzymes that belong to the mutagenic carboxylesterases and are present in the Culicidae family. However, some groups of insecticides such as carbamates and organophosphates perform phosphorylation activities in certain regions

within the organism, inhibiting AChE activity, resulting in an accumulation of acetylcholine in the synapse, which allows the acetylcholine receptor to remain open, leading to paralysis and death (Lionetto *et al*., 2013).

AChE1 and AChE2, encoded by ace-1 and ace-2, respectively, have been identified in different species of mosquitoes, but only the alteration of AChE1 has been related to mosquito resistance to insecticides such as organophosphates and carbamates (Alout *et al*., 2008). Such resistance has been reported in many species of mosquitoes, including *Anopheles gambiae* Giles, 1902; *Culex pipiens* Linnaeus, 1758 (Weill *et al*., 2003; Alout *et al*., 2008), *Culex quinquefasciatus (*Djogbènou *et al*., 2008) *Culex tritaeniorhynchus* Giles, 1901 and *Culex vishnui* Theobald, 1901(Alout *et al*., 2008). Controlling populations of disease-carrying mosquitoes reduces disease transmission. Insecticides such as organochlorines, organophosphates, carbamates, and pyrethroids have been the most widely used groups of insecticides to combat insect vectors (Casida, 1998). Enzymatic mechanisms involved in the hydrolysis of insecticides detoxify the insecticides before they reach the target site of action in the insect (Colovic *et al*., 2013). The phenomenon of insecticide resistance occurs through physiological and behavioral changes that occur in mosquito populations, which evolve in response to repeated exposure to insecticides or other toxins over time. Groups of insecticides with similar modes of action are used in agricultural pest control and have an impact on direct or indirect contamination of mosquito oviposition sites, causing selection that favors insecticide resistance involving changes in the detoxification enzymes present in mosquitoes (Nkya *et al*., 2014).

Biochemical assays are an indispensable tool capable of measuring enzyme activities in mosquito populations (Flores *et al*., 2005). In the Mexican southeast, studies on mosquito resistance are being carried out in the states of Campeche and Quintana Roo to evaluate the activities of the enzymes glutathione S-transferase (GST), esterase, altered acetylcholinesterase (AchE), and monooxygenase in populations of *Anopheles albimanus* (Dzul *et al*., 2007). Little is known about the rational use of synthetic insecticides for pest control. These compounds probably impact acetylcholinesterase enzyme activity in response to insecticides in *Culex quinquefasciatus* populations in this region. This mosquito population is a potential vector of Zika in Mexico (Elizondo-Quiroga *et al*., 2018), and it is therefore necessary to evaluate the mechanisms involved in resistance to insecticides to properly manage this vector. The objective of this research was to identify whether there is insensitivity of acetylcholinesterase to insecticides in populations of *Cx. quinquefasciatus*, collected in urban and rural areas in Yucatan state, Mexico.

MATERIALS AND METHODS

Study area

The study was conducted in the state of Yucatán in southern Mexico and included five urban and five rural areas located inside and outside of the state capital, Mérida (population ~1 million). Mérida has a tropical climate with average daytime temperatures that range between 29 °C in December and 34 °C in July. The rainy season occurs from May to October, which overlaps with the season of greatest abundance of *Cx. quinquefasciatus* although it is present throughout the year (García-Rejón *et al*., 2010**)**. The selected study sites were neighborhoods and communities with a high incidence of dengue virus transmission. The presence of mutations associated with resistance to insecticides has been reported, as has the detoxifying activity of enzymes in *Ae.*

aegypti populations (García-Rejón *et al*., 2018). The urban area included the Vergel-III (East), Center Colony (Center), and Juan Pablo-II (West) neighborhoods, which are located in the city of Mérida, and Umán (southwest), and Kanasin (east), which are part of the metropolitan area of Mérida. The rural areas comprised Hunucma (northwest), Caucel (west), Motul (east), Chochola (southwest) and Xkalakdzonot (east), communities that are located between 9 and 123 km from Mérida (Fig. 1).

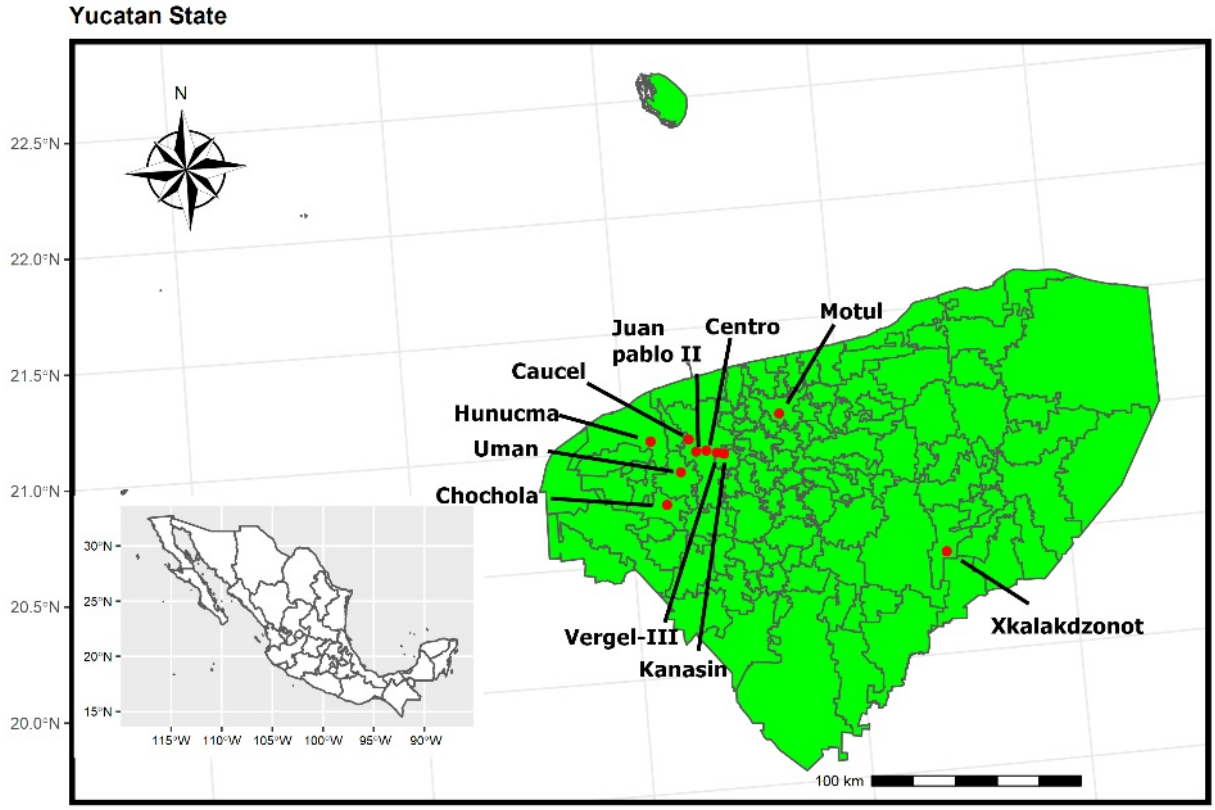


Figure 1. *Culex quinquefasciatus* collection sites in urban and rural areas of Yucatán, Mexico.

Adult mosquito collection

Adults were collected from different houses in the neighborhoods and communities from January to August 2017. Each house was visited once. Collections were made both indoors and outdoors. Adult mosquitoes were collected between 09:00 and 12:00 hrs using CDC style backpack aspirators (Centers for Disease Control and Prevention). Mosquitoes were transported live to the arbovirology laboratory of the Universidad Autónoma de Yucatán Mérida and identified to species using morphological keys (Carpenter & LaCasse, 1955).

Biochemical assays

To determine the activity of insecticide-degrading enzymes associated with resistance, AChE and AChEI enzymes were evaluated in 45 *Cx. quinquefasciatus* females from each collection site. The tests were performed with slight modifications of the Ellman test protocols (WHO, 1998; Brogdon *et al*., 1988). For homogenization of the sample, mosquitoes were macerated individually in an Eppendorf tube (1.5 mL) for each of the sampled areas. In the test, flat-bottom microplates (F96 MaxiSorp Nunc-Immuno Plate (60 / cs) Thermo Scientific were used. Two plates were used per site, one for AchE and another for AChEI. A 25 µl volume of sterile water in the white wells was

used as a control for each microplate, and in this way, all the biochemical tests were run with 25 µl of the homogenate deposited on the transfer plate. Acetylcholinesterase activity was measured in separate microtitration plates using acetylthiocholine iodide. In the first microplate (AChE), 145 µl of Triton solution, 10 µl of DTNB (5,5'-dithiobis-2-nitrobenzoicacid), and 25 µl of ACTH (acetylthiocholine) solution were used; for the insensitive AChE, 145 µl of Triton solution, 10 µl of DTNB, and 25 µl of the ACTH solution was mixed with 6 µl of the stock solution (0.1%) of Propoxur (Chem Service). Both plates were incubated for 60 minutes at room temperature, and the absorbance was read in a spectrophotometer with a 405 nm filter.

Application of surveys on pesticide use practices in the communities

A survey was carried out in the study areas during the period from June 2015 to January 2016 in urban and rural areas of Yucatán, Mexico. A cross-sectional descriptive survey was administered to the head of each household (n = 200) in which *Cx. quinquefasciatus* had been collected. The consent of the heads of family was requested after explaining the objective of the study; the questions were formulated in Spanish and in the Mayan language. Most of the interviewees were men who declared that they worked in nearby agricultural fields. The administration of the questionnaire usually lasted between 10 and 15 minutes, and the survey was related to the frequent use of pesticides in the population for pest and weed control and other commonly used products against mosquitoes, respectively.

Data analysis

A database was made with the absorbance values obtained to subsequently express the results as percentages of remaining activity in the inhibited propoxur samples compared to the control (non-inhibited). Percentages higher than 70% of remaining activity for each mosquito are indicative of resistant homozygotes (RR), 30 to 70% of remaining activity are indicative of heterozygotes (RS) and percentages lower than 30% of remaining activity is indicate susceptible homozygotes (SS) (WHO, 1998). Data could not be normalized by transformation and so were analyzed by using the nonparametric Kruskal-Wallis (KW) test. Knowledge on the use of insecticides was compared in urban and rural areas using the X^2 test on a 2×2 contingency table. When more than 25% of the expected counts were less than five, Fisher's exact test was used. Statistical analyses were performed using IBM SPSS Statistics version 22 for Windows software (IBM Corporation, Armonk, NY).

RESULTS

Population density

For each study site, 45 individuals were analyzed in duplicate for each plate of AChE and AChEI, resulting in a total of 450 individuals. The results of biochemical tests for AChEI were found to be altered in the sites studied, revealing a significant increase in the activity of acetylcholinesterase in some localities with the presence of propoxur (Fig. 2A and 2B). In the urban area, a significant difference was observed between the percentages of acetylcholinesterase activity in the samples from different sites (KW X^2 = 50.65, *d.f.* = 4, $P < 0.001$). A greater activity of this enzyme was estimated in mosquitoes collected in Vergel III (53%), Center Colony (45%), and Uman (42%). In the rural areas, a statistically significant difference in the remaining activity of the enzyme was also observed across these sites (KW X^2 = 19.72, *d.f.* = 4, $P = 0.001$). However, the percentages of AChEI

activity measured as a function of its remaining activity also varied in the municipalities of Motul (30%), Hunucma (27%) and Xkalakdzonot (25%) (Table 1).

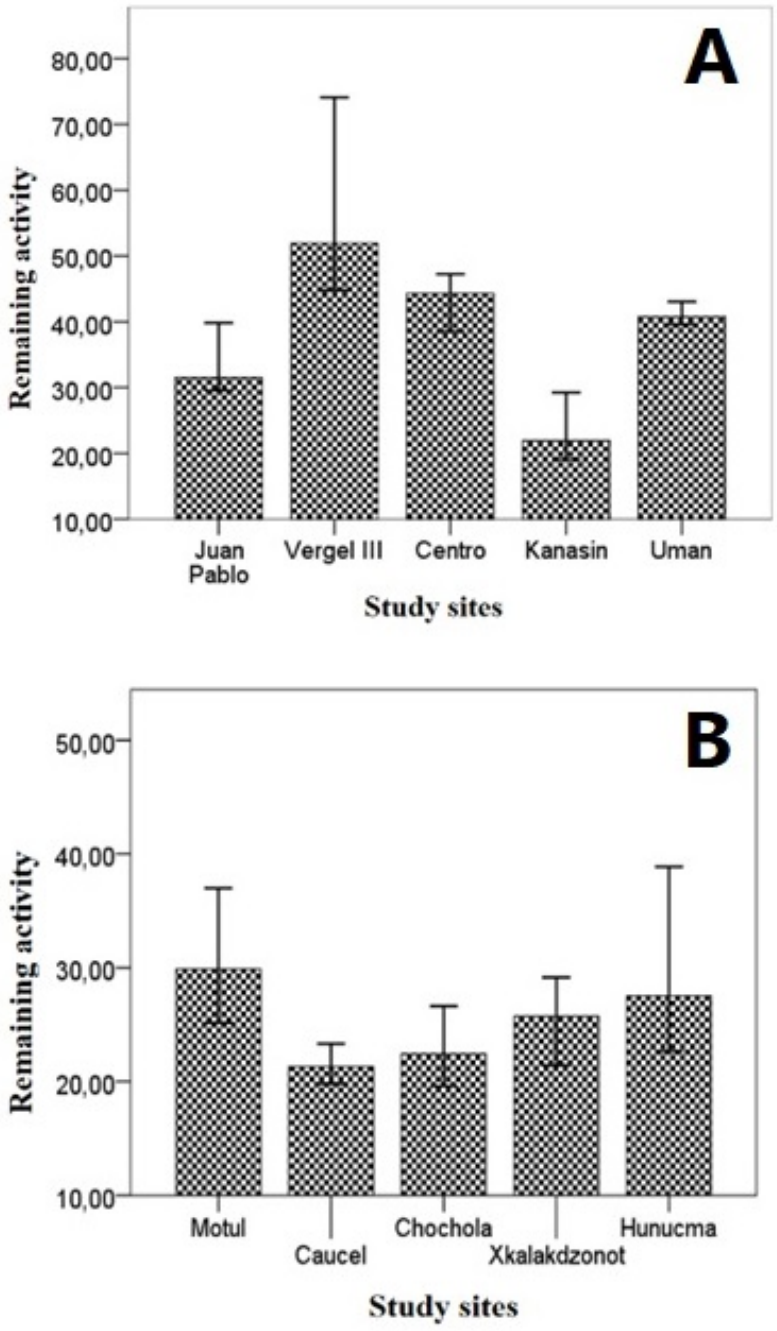


Figure 2. Remaining acetylcholinesterase activity in *Culex quinquefasciatus* collected in (A) urban and (B) rural areas of Yucatán in the presence of propoxur. Error bars indicate 95% CI.

Implications of chemical insecticides on acetylcholinesterase activity levels

A total of 100 surveys were conducted in five sites in the urban area and 100 in the rural area on the domestic use of pesticides in the region, for activities such as gardening, agriculture and pest control, including mosquitoes. The information was collected from 20 individuals per site. The data

obtained from each of the surveys were used to calculate the percentage of products used to control insects, likely to be related to the indirect impact of such uses on insecticide resistance.
The majority of respondents reported the presence of mosquitoes inside their houses (Table 2). Ninety-one percent of the respondents indicated a positive response, and a very low percentage in the urban area gave a negative response (9%). One hundred percent of the respondents in the rural area reported finding mosquitoes inside their homes, therefore, they made use of some pesticides. Eighty-three percent of respondents in the urban area reported using products such as aerosol insecticide sprays, ecological repellents (i.e., clove with apple cider vinegar, clove with lemon, clove with Mennen® oil and alcohol, citronella cream), Off Family® repellent, and impregnated wristbands. Only 57% of respondents in rural areas reported use of these products, perhaps due to their high cost. In reference to aerosol insecticides, 90% of the people surveyed in urban areas mentioned having used this type of product; however, the consumption and use of these products increased during the rainy season. Likewise, only 66% of people in rural areas use this type of product to combat mosquitoes (Table 3). Regarding the use of herbicides for weed control, 58% of the respondents in the urban area indicated having used this type of chemical product while 39% of the respondents in the rural population used these products. In reference to knowledge about the use of Temephos (an organophosphate) to control mosquito larvae, 28% of the respondents in the urban area used this product, and 43% of the respondents in the rural area had access to this chemical and also applied it in their homes (Table 2).

Table 1. Number of individuals and prevalence (%) of genotypes showing a reduction in the acetylcholinesterase response after 0.1 % propoxur inhibition in populations of *Culex quinquefasciatus* from urban and rural localities in and around Mérida, Yucatán.

Localities	Susceptible individuals	Heterozygous individuals	Resistant individuals
Urban areas			
Col. Juan Pablo II	18 (42.9%)	23 (54.8%)	1 (2.4%)
Col. Vergel III	7 (16.6%)	19 (54.2%)	16 (38.1%)
Col. Center	8 (19.0%)	31 (73.8%)	3 (7.1%)
Uman	1 (2.4%)	40 (95.2%)	1 (2.4%)
Kanasin	27 (64.3%)	14 (33.3%)	1 (2.4%)
Rural areas			
Hunucma	25 (59.5%)	6 (14.3%)	10 (23.1%)
Motul	21 (50.0%)	20 (47.6%)	1 (2.4%)
Caucel	35 (83.3%)	7 (16.7%)	0
Chochola	30 (71.4%)	12 (28.6%)	0
Xkalakdzonot	29 (69.0%)	11 (26.2%)	2 (4.8%)

Susceptible individuals were those that showed <30% inhibition. Heterozygous individuals were those that showed 30-70% inhibition. Resistant individuals were those that showed >70% inhibition.

Table 2. Frequency of survey responses related to the use of insecticides and herbicides.

	Urban		Rural		Statistic	P-value
	Yes	No	Yes	No		
¿Are there mosquitoes in your house?	91	9	100	0	-	0.006*
¿Do you use any commercial repellents?	83	17	57	43	16.095	<0.001**
¿Do you spray insecticides to reduce mosquitoes?	90	10	66	34	16.783	<0.001**
¿Do you use herbicides to reduce weeds?	58	42	39	61	7.227	0.007**
¿Do you use temephos to reduce mosquito larvae?	28	72	43	57	17.207	<0.001**
Totals	350	150	305	195		

* Fisher's exact test

** X^2 test

Table 3. Products commonly used to control mosquitoes and insect pests in urban and rural areas of Yucatan.

Zone	Products used for vector control	Other chemicals used locally
Urban	**Baygon®** pyrethroids: cyfluthrin, transfluthrin, praletrin; propoxur (carbamate) and chlorpyrifos (organophosphate). **Alfadex®** (cypermethrin) for the control of livestock ectoparasites **Raid casa y jardín®** (pralethrin, fenothrin). **H24®** (tetramethrin, cyphenothrin). **Insecticide of biological origin**	**Gramoxone®** (paraquat dichloride herbicide, bipyridylium family). **Cerillo®** (paraquat dichloride + diuron herbicide). **Dragon®** (picloram, trisopropandamide salt) **Secador®** (paraquat dichloride herbicide). **Velfosato®** (glyphosate isopropylamine salt).
Rural	**Baygon®** pyrethroids: cyfluthrin, transfluthrin, praletrin; propoxur (carbamate) and chlorpyrifos (organophosphate). **Raid casa y jardín®** (pralethrin, phenothrin). **H24®** (tetramethrin, cyphenothrin). **Rayoback®** (permethrin) **Aquarreslin®** **(**permethrin, esbiol and esbioalethrin) **OKo insecticide®**	**Plancha** **Antorcha®** (paraquat dichloride). **Secador®** (paraquat dichloride). **Gramoxone®** (paraquat dichloride). **Cerillo®** (paraquat dichloride + diuron). **Diablito®** (2,4-D dimethylamine salt herbicide). **Velfosato®** (glyphosate isopropylamine salt).

DISCUSSION

The use of chemicals and house spraying for the control of disease-transmitting insects has been one of the strategies for the reduction of adult mosquito populations. However, control interventions using insecticides are affected by the selection mechanisms associated with insecticide resistance. In our study, it was possible to perform biochemical assays of the AChEI enzyme in populations of *Cx. quinquefasciatus* associated with resistance to organophosphate and carbamate insecticides, which are the target for this group of insecticides (Djogbèou *et al*., 2008). It is worth mentioning that the results from urban areas (Vergel II, Col. Center, and Uman) demonstrate the role played by AChEI in the development of resistance to propoxur (carbamate). Similarly, resistance to organophosphates (chlorpyrifos and malathion) has been reported in the State of Sonora, Mexico in species of *Ae. aegypti* (Villegas, 2021). Carbamates are used in vector control programs in Mexico due to their low toxicity to mammals and broad-spectrum residual control characteristics. They are used widely in pest control, including arbovirus vectors, and behave similarly to organophosphates in biological systems. Studies conducted by Loroño-Pino *et al*. (2014) in the city of Mérida and other communities in the state of Yucatán, showed that 87% of the human population applied vigorous measures for pest control, that included mosquitoes such as *Ae. aegypti* and *Cx. quinquefasciatus* that are abundant in the region during the rainy season (García-Rejón *et al*., 2008).

In the indoor habitat, *Cx. quinquefasciatus* is probably exposed to chemicals such as carbamatos in combination with cyfluthrin and organophosphates (dichlorvos) (Loroño-Pino *et al*., 2014). These findings help us understand the insensitivity of acetylcholinesterase to propoxur in certain sampled areas. Indeed, resistance to propoxur and malathion has been documented previously in populations of *Cx. quinquefasciatus* (Low *et al*., 2013). It is important to note that more than 80% of the surveyed residents in urban and rural areas had carried out some measure to control insects in their homes involving the use of pesticides for urban and agricultural use, herbicides, and biological and homemade products that they implemented. Importantly, the responses of the respondents, probably do not match the trade names of the products that are usually used in the regions, since evidence was not obtained in physical form. We hypothesize that there is probably additional environmental selection on *Cx. quinquefasciatus* populations caused by the use of these compounds as they may accumulate in bodies of water that become are used for oviposition and larval mosquito development (Czeher *et al*., 2008). This is because the products that are usually used in agriculture belong to the same chemical classes and present the same target and mode of action in insect vectors (Overgaard, 2006; Suman *et al*., 2010; Tanley *et al*., 2010; Osta *et al*., 2012).

Some urban areas that presented high percentages of AChEI individuals probably were contaminated with agrochemical residues that are filtered into drains during the washing of application equipment (Corvel *et al*., 2007), and other pollutants that people use in their daily activities in drains such as storm drains that mosquitoes use for their development (Manrique-Saide *et al*., 2012). These drains are treated before the rainy season by health personnel on a frequent basis in the region, applying larvicides such as temefos, and could also be implicated in selecting for propoxur resistance. There is a possibility that these chemical residues could favor the development of other resistance mechanisms in mosquito populations. It has been shown that insects can develop tolerance to insecticides by modulating their detoxification system with enhanced activities of other enzymes (Li *et al*., 2009). The development habitats of *Cx.*

quinquefasciatus larvae often contain high amounts of organic matter that is rich in dissolved plant chemicals that serve as a food source for larvae. Xenobiotic compounds affect the metabolism of larvae and could contribute to their tolerance of insecticides (Riaz *et al*., 2009; Kim and Mutiri, 2012; Poupardin *et al*., 2012). As such, it is important to understand the ecology of this vector in southeastern Mexico, where urban and rural areas both present ideal breeding sites for mosquito reproduction.

In conclusion, our study provides for the first time the detection of acetylcholinesterase insensitivity to propoxur in populations of *Cx. quinquefasciatus* in urban and rural areas in the State of Yucatán. Resistance to insecticides in the State was previously reported in populations of *Ae. aegypti* (López *et al*., 2014) and pyrethroid resistant alleles are known to be present in *Cx. quinquefasciatus* (Chi-Chim *et al*., 2018). The use of chemical products for pest control, both urban and agricultural, likely influences the tolerance to insecticides used in mosquito control programs in Mexico. We consider that it is important to understand the environmental factors that affect the behavior and response of mosquitoes to insecticides for the efficient design of vector control programs.

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