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Acetylcholinesterase Activity in Brain Tissue of *Profundulus punctatus* and *Poecilia butleri* from a Watershed of Southern Mexico

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ABSTRACT

Organophosphate and carbamate compounds are used in agriculture and husbandry in the Coatán watershed and have been widely used in maize, coffee and banana crops, where they are carried to the water bodies. These compounds inhibit the acetylcholinesterase enzyme activity in aquatic organisms, which is considered a biomarker for organophosphate and carbamates in the environment. The objective of this work was to evaluate the brain acetylcholinesterase activity inhibition in the fish species *Profundulus punctatus* and *Poecilia butleri* from Coatán watershed. The river was subdivided according to environmental and anthropic activities: upper, middle, transition and lower zones. Since none of the species are distributed all along the river, two species were collected, *Profundulus punctatus* for upper and middle zones and *Poecilia butleri* for transition and lower zones. The collecting was performed in two seasons, high and low precipitation. The enzyme activity was measured in the fish brain tissue and the analysis results revealed that the activity decreased from high to low precipitation in all river zones, enzymatic inhibition in upper zone ranging from 19.27 in high precipitation to 40.48% in low precipitation seasons and in the middle zone from 22.70 to 38.12% in high and low precipitation respectively. In the transition and lower zones, the inhibition ranging from 55.96 to 72.82% and 52.04 to 64.48% in high and low precipitation respectively. The inhibition of the enzymatic activity in brain tissue on *Profundulus punctatus* and *Poecilia butleri* of the Coatán River could be considered as indicator of pesticides in the aquatic environment.

INTRODUCTION

Organophosphate (OPs) and carbamate (CBs) compounds are commonly used for pest control in agriculture, husbandry and urban dwellings; their persistence in the environment is relatively low and does not biomagnify [1,2]. However, inadequate use of these substances has led to find them in natural water bodies, soil and food [3].

The enzyme acetylcholinesterase (AChE) is responsible for regulating nervous signals by hydrolyzing acetylcholine (ACh) in choline and acetic acid, avoiding its accumulation [4]. The activity of this enzyme decreases in presence of OPs and CBs due to their phosphorus and carbon atoms which affecting its active site, impairing its binding to ACh, rendering the enzyme phosphorilated or carbamated correspondingly. The neurotransmitter accumulation in the synapses affects the central and peripheral nervous system as well as neuromuscular functions, resulting in neurological disorders such as sensory and behavior disturbances lack of coordination, motor and respiratory function depression that eventually lead to death. Studies in crustaceans, fish [1,2,5], birds [6], mammals [7], and even in humans [8], have shown that the AChE activity decreases as a consequence of exposure to OP and CBs.

In aquatic ecosystems, fish are particularly vulnerable to the presence of OPs and CBs because their entire life cycle occurs in water [9]. For example, the effect of 0.05 mg/L of OPs over the AChE activity in *Oncorhynchus mykiss*, *Gambusia yucatana, Catostomus occidentalis* (in muscles) *and Lagodon rhomboides* (in brain), renders an enzymatic activity decrease of up to 60%

after 72-96 hours of exposure [1,2,4,10,11]. In brain tissue of *Seriola dumerelli, Oreochromis niloticus* and *Channa striatus* the enzymatic inhibition of AChE reached levels of 75% [12-14].

The Coatán watershed located in México-Guatemala border (northern Central America), as other watersheds of Southeast of México and Central America is undergoing important changes in the land use due to the constant growth in agricultural activities [15], around 65% of its surface are cultivated lands [16], where large amounts of chemicals are used, such as O,O-Diethyl O-[4-methyl-6-(propan-2-yl)pyrimidin-2-yl] phosphorothioate, 2,2-Dimethyl-2,3-dihydro-1-benzofuran-7-yl methylcarbamate, Diethyl 2-[(dimethoxyphosphorothioyl)sulfanyl]butanedioate, N-(phosphonomethyl)glycine and O,O-Diethyl S-[(ethylsulfanyl)methyl] phosphorodithioate in mango, banana, maize, coffe and soybean plantations [17]. Only in the municipality of Tapachula (main municipality in the Coatán watershed), during 2010-2015, around 1.875 tons only of N-(phosphonomethyl) glycine were used [18]. The use of these types of compounds is permitted due to their low environmental persistence, which has led to their extensive use throughout the watershed [17].

Since most of the area from the Coatán watershed is cropland where the use of OPs and CBs is extensive, their presence in the aquatic environment may endanger the ecosystem and affect the living organisms in the area. The purpose of this study was to determine the AChE inhibition in the brain tissue of fish *Profundulus punctatus* and *Poecilia butleri* from the Coatán Watershed.

MATERIALS AND METHODS

Area of study

The Coatán watershed is located in the Southeast of Chiapas state of Mexico, with a surface of 733 Km^2; part of its territory (30%) is shared with Guatemala, **(Figure 1).** It is located within the coordinates 15° 16' 36" - 14° 46' 12" North; and 92° 31' 12" - 92° 07' 36" West [19]. The topography is characterized by three zones: The upper zone (UZ), highlands of the Sierra Madre of Chiapas; middle zone (MZ), lands of the lower parts of the Sierra Madre (hills zone); and the Lower Zone (LZ), coastal flat lands [20].

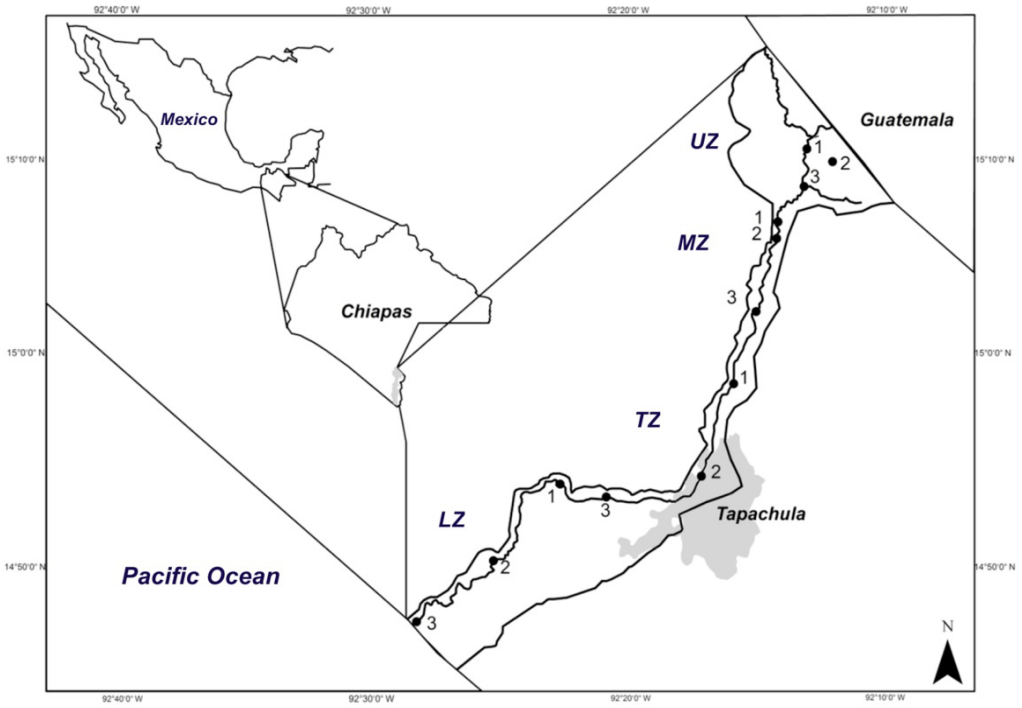

Figure 1. Location of the Coatan River and Coatan Watershed, zones and sampling sites. UZ= upper zone, MZ= middle zone, TZ= transition zone and LZ= lower zone. 1, 2, 3= sampling sites11.

There are two main seasons in the area, the high precipitation season (HP), which begins in June and ends in November with average monthly rainfalls of 2990.33 mm, and the low precipitation (LP) season that begins in December and ends in May with average monthly rainfalls of 73.6 mm [21].

For the research purposes, the Mexican portion of the watershed was divided into four main regions according to the dominant land use and vegetation type; the UZ: pine-oak forest, medium sub-perennial or low perennial forest and low deciduous forest, the main crops are corn, beans and coffee; the MZ: coffee plantations are dominant with incipient portions of medium sub-perennial forest, low deciduous forest; transition zone (TZ): urban area (Tapachula City), and low zone (LZ): dominated by seasonal crops such as banana, soy, corn, sesame among others [22]. In each zone, three sampling sites were established in the mainstream the Coatán River, the first one before the most active area of the corresponding zone, the second one in front of the active area and the third one past the area **(Figure 1).**

Organism collection

Since none of the fish species are distributed along the entire watershed, two species were collected over the mainstream, *Profundulus punctatus* in UZ and MZ and *Poecilia butleri* in TZ and LZ. The collecting was performed during the HP and LP seasons using different methods such as fish traps, cast nets and spoon nets. Once the fish were trapped, they were place in ice and transported to the laboratory for their immediate analysis. The fish were identified to species level according to Alvarez del Villar [23] and Miller [24]. Since it was not possible to maintain the fish species in the lab to obtain the basal values, these values were obtained from individuals of UZ2 for *P. punctatus* (17 individuals), because this site were considered less impacted due to clear running water and the original riparian vegetation in its riviera. For *P. butleri* the individuals were collected from site TZ3 (7 individuals) a pond of spring water; both groups of individuals recorded the highest enzymatic activity during HP season.

Enzymatic analysis

The AChE activity determination was conducted following the method described by Ellman [25], modified by Hill and Fleming [26]. The organisms were dissected to obtain the brain tissue, which was homogenized with Tris buffer pH 8 (1 ml/100 mg tissue). Afterwards, 10 µl of the mixture were added 1.5 ml of 5-5´-dithiobis-2 (nitrobenzoic acid) and 50 µl of acethylcholine iodide substrate. The reaction was monitored every 30 seconds for three minutes at room temperature (24 ± 1°C). The readings were recorded three times in a HACH DR5000 spectophotometer at 405 nm. The data units were displayed in µmol acethylthiocholine/ min/g tissue.

Statistical analysis

During the sampling collection, no fish were found in some of the selected sites. For statistical porpoises the unbalanced model was compensated using Boostrap re-sampling method [27]. To achieve this, the data from the same zone and the nearest collecting site were used. The data analysis was performed according to the sampling design proposed by a nested multifactor General Linear Model (GLM), using a type IV sum of squares since the number of observed data was not similar in each factor level. The mean difference between factor levels was verified using a Duncan test [27]. Both the general analysis and the later tests were done using a 0.95 confidence interval. The calculations were obtained using Statistica 7 software StatSoft.

RESULTS

Upper and middle zones

A total of 128 adult fish of *P. punctatus* were collected in the UZ (57 individuals) and MZ (71 individuals) that corresponded to 11 males and 117 females. In the site UZ1, no individuals were collected in any seasons.

The statistical analysis indicated that the AChE activity shows significant differences between two climatic seasons ($F_{(1,142)}$=25,116, p=0.0000), with higher values in the HP. The UZ individuals displayed higher enzymatic activity during the HP season **(Figure 2).** The MZ enzymatic activity did not show differences between seasons ($F_{(2,142)}$=5,2044, p=0.0659); similarly, there were no differences among the sites in both seasons.

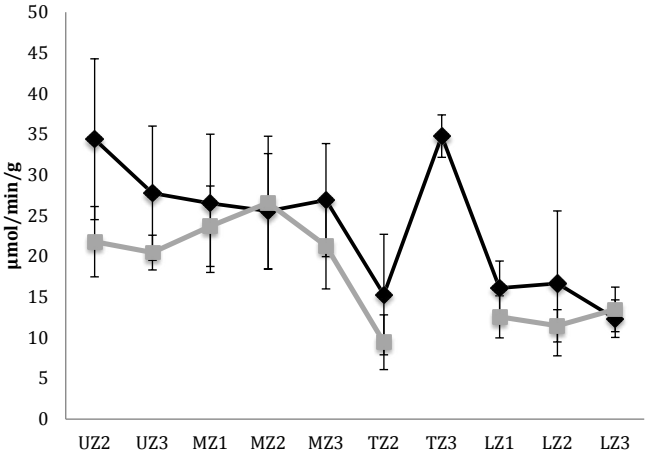

Figure 2. Mean values of AChE activity in fish brain tissue from the different zones and sites of the Coatán River. In the UZ (upper zone) and MZ (middle zone) the analyzed tissues corresponded to *Profundulus punctatus.* In the TZ (transition zone) and LZ (lower zone) tissues were obtained from *Poecilia butleri.* The dark line corresponds to the high precipitation season (HP), the gray line corresponds to the low precipitation season (LP). The numbers next to the acronyms refer to the site number.

The AChE activity at site level in the UZ ranged from 20.47 ± 2.14 µmol/min/g (UZ3) during LP to 34.40 ± 9.88 µmol/ min/g in UZ2 **(Figure 2)** during the HP season; in this last site, the highest enzymatic activity for *P. punctatus* was observed and hence considered as the reference value for the inhibition percentage calculations. The enzymatic activity for the MZ ranged from 21.29 ± 5.28 µmol/min/g in MZ3 during the LP season to 26.92 ± 6.95 µmol/min/g during HP season **(Figure 2).** There were no significant differences found at this level ($F_{(8,142)}$=1,4643, p=0.17539).

Enzymatic inhibition of AChE in *P. punctatus* was recorded in all the UZ and MZ sites during both seasons **(Figure 3).** The lowest inhibition was observed in the UZ during the HP season with a 19.27% AChE inhibition, whereas the highest inhibition was recorded in the same zone during the LP season with a 40.48% decrease.

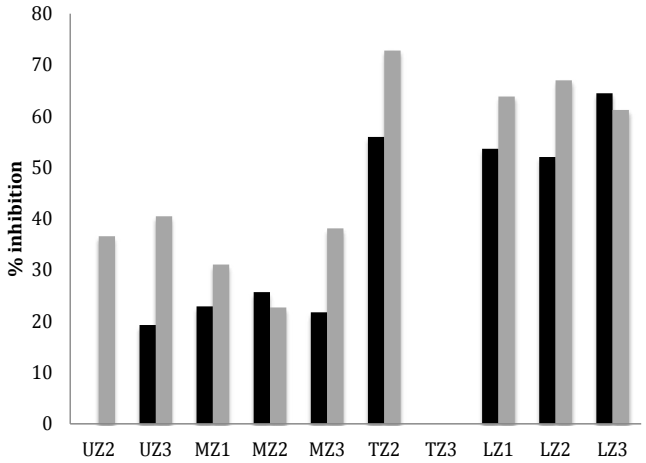

Figure 3. Inhibition percentages of AChE in fish brain tissue from the different zones and sites of the Coatán River. In the UZ (upper zone) and MZ (middle zone), the analyzed tissues corresponded to *Profundulus punctatus.* In the TZ (transition zone) and LZ, (lower zone) tissues were obtained from *Poecilia butleri.* The dark bars corresponds to the high precipitation season (HP), the gray bars corresponds to the low precipitation season (LP). The numbers next to the acronyms refer to the site number.

Transition and lower zones

A total of 160 *P. butleri* fish were collected for the TZ (70 individuals) and LZ (90 individuals) of which 27 were males and 133 females, averaging 13 individuals per site. No individuals were found in TZ3 during LP.

There were significant differences between climatic seasons ($F_{(1,192)}$=65,190, p=0.00000), with less activity during LP. The mean difference test showed that TZ differs between seasons ($F_{(2,192)}$=11,090, p=0.00003). The LZ also differs between seasons with the lowest enzymatic activity during LP.

The enzymatic activity at site level for TZ ranged from 9.45 ± 3.36 µmol/min/g (TZ2) during the LP season, to 34.78 ± 2.61 µmol/min/g (TZ3) during the HP season **(Figure 2).** The latter site presented the highest enzymatic activity for *P. butleri*, so this value was used as reference. The enzymatic activity for the LZ sites ranged from 11.47 ± 1.98 µmol/min/g during the LP season, to 16.68 ± 8.9 µmol/min/g during the HP season, both values were recorded at the same site (LZ2). The TZ and LZ revealed inhibition percentages above 50% in both seasons and in all the sites. The statistical analysis did not show significant differences among sites.

Enzymatic activity inhibition of AChE was recorded in all the TZ and LZ sites. The lowest inhibition percentage was observed in LZ2 with 52.04% during HP, while the highest inhibition was recorded in site TZ2 during LP season with 72.82% **(Figure 3).**

DISCUSSION

Since there is no other important source of compounds that could affect AChE activity like Phenanthrene, Piperidines, etc, the extensive use of OPs and CBs in the watershed can be considered as the main source of enzymatic inhibitors in the aquatic system of Coatán watershed.

Direct measurement in river water was not performed in this study; however there is evidence that OPs and CBs are present in the region. Hernández-Hernández [28] estimated the risks to aquatic ecosystem by pesticides commonly used in a crops of Huehuetán watershed, west side of Coatán watershed, in this study Manganese ethylene-bis-dithiocarbamate, Dxiethyl 2-[(dimethoxyphosphorothioyl)sulfanyl]butanedioat and other pesticides were considered as high exposure to aquatic organisms due to frecuent and extensive application. Hernández-Romero [15] found O, O-dietil O-3,5,6-trichloropyridin-2-ilfosforotioato in water samples of a wetland east side of Coatán watershed.

The brain AChE enzymatic activity was inhibited in both fish species in all the zones and collecting sites during both seasons. The overall inhibition values for *P. punctatus* (from 19.27% to 40.48% in UZ and MZ) and for *P. butleri* (from 52.04% to 72.82% in TZ and LZ) are similar to those reported for *Mugil cephalus* [29] and *Cyprinus carpio* [30] which indicated an activity decrease up to a 60%, which was related to the high agricultural activity, drainage and irrigation areas; also, inhibition was more pronounced during low precipitation season, a similar trend observed in the present study [29,30]. Studies in *Gambusia yucatana* reported inhibitions in AChE activity up to 48% and displayed the same seasonal behavior pattern [31].

Controlled exposure to OPs and CBs in *Oreochromis niloticus, Gambusia affinis* and *Clarias gariepinus* revealed inhibition values of 50%, similar to the ones in the present study [32-34]. The inhibition results obtained evidenced this type of compounds are present in the Coatán watershed.

In order to OPs and CBs reach the aquatic ecosystem, it is not necessary to pour them directly in the river; these compounds can be transported and moved through the environment by different means. In soils with high contents of organic matter, the pesticide movement is low, due to the humic acids, which retain them for a longer period of time. However, when humidity in the soil increases, adsorption decreases and the compounds move through the soil from the high concentration areas to the low concentration ones [35]. Pesticides in agricultural soils with low organic matter levels can be easily transported by water run-off to secondary streams, which eventually reach the main stream, especially during the rainiest seasons. This type of movement can be one major way for these compounds to reach the rivers. In the HP season, the river water flow increases, especially during the rainiest months (September and October), promoting the pesticide dilution into the water. Although water runoff can move compounds to the main stream, the water volume increase (and its flow speed) also promotes its dilution [28,36], therefore, it is probably that this process can reduce the chances of contact between pesticides and the organisms that inhabit Coatán River, and it may explain the lower AChE inhibition. It is noteworthy to mention that fish during the high water flow tend to seek shelter in zones away from the stream turbulences, and could help them avoid exposure to these compounds (such behavior was observed during the field collecting).

The differences in enzymatic activity between UZ and MZ were constant in both seasons, which could be attributed to the agricultural activities that occur in both areas, since the UZ supports subsistence agriculture (maize and beans mainly) with pesticide applications limited only during the production season, in addition, the crops are mainly established in high slope, eroded, low fertility soils with few agricultural treatment [37]. The AChE inhibition levels in this area also could indicate that OPs and CBs could be move from Guatemala croplands and are likely to be present in the UZ but at relative low concentrations.

In the MZ, the cropland surface is larger and support perennial crops such as coffee, which receive constant maintenance; consequently, pesticides are used throughout the year. The obtained results are consistent with the use of pesticides in the area, presenting higher AChE inhibitions than in the UZ.

During the LP season, the pesticide transport mechanism is different. Volatilization can be one of the main processes in which pesticides can move in the environment [38]. The volatilization process occurs in two stages, in the first one, there is a pesticide movement towards the soil surface through evaporation (this process is faster in naked soils); in the second stage, the compounds enter the atmosphere in the form of gases, where they are transported by the winds to be deposited in any other place. Wind transport can also occur immediately after pesticide applications and depends on the physical and chemical properties of the product (vapor pressure, water solubility), environmental conditions (temperature, wind speed) and soil features (organic matter content) [39]. In this way, pesticides can also reach river streams besides water run-off.

Organophosphate and Carbamate pesticides degrade faster due to microbial decomposition, fotochemical reactions, volatilization, hydrolysis and high temperatures [40]. Pesticide breakdown can lead to the formation of even more toxic metabolites than the original compound and can be more easily absorbed by tissues as temperature increases, leading to even stronger AChE inhibitions. For example, studies in crabs and aquatic insects showed a proportionally direct relation between OPs absorption (0, 0-diethyl 0-3, 5, 6-trichloropyridin-2-il phosphrothioate and 0,0-dimethyl 0-4-nitrophenyl phosphrothioate) as the water temperature increased [41-43]. During the LP season, a large part of the soil from the seasonal crop areas is exposed to winds, solar radiation and high temperatures typical from this season. Additionally the water volume of the Coatán River decreases, which can increment the probability of exposure to these compounds. These environmental conditions can magnify the pesticide effects and their probability of exposure; this could be responsible for the increase in the enzymatic inhibition percentages found in fish during the LP season.

During LP season, the TZ y LZ showed highest inhibition values. The 72.8% of inhibition founded in TZ could be related to the use of pesticides in Tapachula City and inadequate pesticide container disposal [44]. The high inhibition observed in LZ could be related to extensive agriculture activities, in this zone, the agriculture is the dominant activity and uses nearly the 97% of this area, and some crops are perennial like mango, banana and cocoa and some temporal like soy, sesame and maize, all of them used pesticides.

It is important to mention that studies done in fish and birds have determined that inhibitions above 20% indicate exposure to AChE related pesticides, inhibitions above 50% indicate important negative effects, and some above 70% are considered life threatening inhibitions [45-47]. According to this, the inhibitions observed in fish from the UZ and MZ of the Coatán River indicate that they are being exposed to OPs and CBs with reversible effects if the exposure disappears. However, the inhibitions observed in the TZ and LZ indicate that the organisms are being exposed to life threatening pesticide concentrations, which could eventually kill them, since those inhibition levels are irreversible.

Finally, the basal brain tissue AChE values for *P. punctatus* (34.40 ± 9.88 µmol/min/g) and for *P. butleri* (34.78±2.61 µmol/min/g) were similar despite these fish belong to different families, which may indicate that the brain AChE values can be similar for all species from the order Cyprinodontiformes, suggesting that the use of any species of this order could be used as biomarkers of exposure to OPs and CBs, but further proof is needed.

CONCLUSION

The results from this study revealed enzymatic activity inhibition in *Profundulus punctatus* and *Poecilia butleri,* which indicates the presence of Organophosphate and Carbamate compounds in the aquatic environment of the Coatán watershed. Regardless of the climatic season, the inhibitory effect over the brain tissue acetylcholinestherase increases in the areas with higher agricultural and urban activity.

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CONFLICT OF INTEREST

The authors state that they have no interest conflict.

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