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JEB
Journal of Environmental Biology

ISSN: 0254-8704
CODEN: JEBIDP

Use of cholinesterase activity as a biomarker of pesticide exposure used on Costa Rican banana plantations in the native tropical fish *Astyanax aeneus* (Günther, 1860)

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Publication Info

Paper received:
08 March 2013

Revised received:
12 June 2013

Accepted:
05 September 2013

Abstract

In Costa Rica, thousands of tones of agricultural pesticides have been used for decades and their use is continuously increasing due to intensive and expanding production of coffee, pineapple, rice, ornamental plants and bananas. The objective of this study was to evaluate whether choline esterase (ChE) activity could be used as a biomarker of exposure to pesticides in the Costa Rican native fish *Astyanax aeneus* (characidae). Three methods used in order to evaluate the ChE biomarker were as follows: Laboratory studies where *A. aeneus* was exposed to organophosphate pesticide (ethoprophos); *In situ* 48 hr exposure assessment using caging experiments with fish exposed upstream and downstream of banana plantations and ChE activity estimation of in fish captured directly at sites with different degrees of pesticide exposure. Results from the laboratory studies showed that ChE activity in both brain and muscle tissue was significantly lower in fish exposed to ethoprophos than in controls. Fish from the caging experiments showed no difference in ChE activity neither in brain nor in muscle tissue between the four tested sites and was attributed to the short duration of the exposure. A significant difference in ChE activity was determined in muscle of fish captured from Laguna Madre de Dios compared to fish from Canal Batán. Although our laboratory results revealed that ChE activity in *A. aeneus* was highly responsive to ethoprophos, results from field experiments were less conclusive and showed that the captured fish showed large variability in ChE activity and that more research is needed before ChE activity can be used as reliable biomarker of pesticide exposure.

Key words

Astyanax, Biomarker, Cholinesterase activity, Ethoprophos

Introduction

In Costa Rica, agriculture is one of the main economic activities and the most important export revenue. Banana is the major export agro-product, mostly to Europe and North America (Arce, 2008). Banana is produced in large monocultures in a very intensive manner. This kind of agriculture combined with the tropical environmental conditions of high humidity, rain and temperature, favors the presence of pests, leading to extensive use of pesticides (Ramírez *et al.,* 2009). The most commonly used pesticides in banana plantations are fungicides, nematicides and insecticides. The organophosphate Ethoprophos is one of the main insecticides applied on banana plantations, which, for several years, has been within the top ten of the main pesticides imported to Costa Rica (Ramírez *et al.,* 2009). Residues of this pesticide have frequently been detected in water and sediment samples collected close to the plantations, and it is suspected to be the cause of large fish kills regularly reported in these areas, especially following heavy rainfalls (Castillo *et al.,* 2000).

The impact of pesticide used in plantations at the Caribbean zone of Costa Rica on non target species in adjacent streams has been demonstrated using chemical analyses of pesticide residues in water, sediment and biota, changes in macro invertebrate communities and bioassays with sensitive organisms such as small crustaceans and algae (Castillo *et al.,* 1997; de la Cruz *et al.,* 2003). These bioassays generally use effect endpoints such as mortality, impaired reproduction, or absence of sensitive species in the community. The biomarker approach, however, used intensively in current environmental monitoring (Rendón-von Osten *et al.,* 2004; Sarkar *et al.,* 2006; de la Torre *et al.,* 2007; Sanchez *et al.,* 2007), offers the advantage to detect early sub-lethal effects of pollution on living organisms, thus potentially enabling their use as an early warning signal, i.e. before more severe effects such as mortality may occur. This makes the exposure evaluation more sensitive and gives the possibility to detect the effect of lower pollutant concentrations.

The use of biomarkers in ecotoxicology and in environmental risk assessment has been criticized. Most of the arguments, question the difficulty of linking individual responses to effects at the population and community levels; the cost-effectiveness of the analysis and the usefulness of the information generated by biomarkers in the environmental risk assessment frame (Forbes *et al.,* 2006). However, biomarkers are intended to give information about exposure to pollutants at the individual level. Biomarkers of exposure can be quite useful as an early warning signal before effects at more ecologically relevant levels (populations or communities) can be observed (Guilhermino, 2007).

Inhibition of cholinesterase activity has been intensively used as a biomarker. It is one of the better characterized biomarkers applied in environmental studies, showing a specific response to organophosphate and carbamate pesticides (Gruber and Munn, 1998; Thomson, 1999; Pathiratne *et al.,* 2008). In this regard, AChE inhibition is considered as a biomarker that could even replace chemical analysis in detection of exposure to pesticides (Peakall, 1992). Considering the intensive use of organophosphates in banana production, ChE inhibition appears to be an ideal biomarker to evaluate exposure from pesticides to non-target organisms in the area. This biomarker may also help in establishing causality between pesticide residues detected in field and toxicity effects observed in laboratory experiments which might be an additional useful tool in ecological risk assessments.

Acetylcholine esterase is an enzyme that inhibits the neurotransmitter acetylcholine after its release into the synaptic cleft during transmission of nerve impulses. Thus, as a marker, ChE activity can be measured in different tissues of an organism. Some tissues such as brain and muscles contain more nerves than other tissues and are thus are more suitable for measurement of ChE activity. There is, however, a large variability in the enzyme activity of these tissues that is not attributable to toxic effect, but rather to other natural factors such as size or age. In *A. aeneus* it has been observed that ChE activity in muscle decreases with the size of fish, whereas ChE activity in brain does not .

Use of biomarkers on native species is a common and recommended practice in ecotoxicology (Wijeyaratne and Pathiratne, 2006; Whitehead *et al.,* 2005). In Central America, however, there are, so far, no fish species that has been identified as a suitable model organism for biomarkers in the area. *Astyanax* aeneus is a native species in Costa Rica which presents some advantageous characteristics in this regard: it is present in the whole country in various water systems, ranging from estuarine to fresh water; has a convenient size (6 – 14 cm) and has abundant populations (Bussing, 2002). In addition, species of the genus *Astyanax* has already been used to study ChE inhibition in response to exposure to hydrocarbons (Akaishi *et al.,* 2002) and biocides (Oliveira Ribeiro *et al.,* 2002) in South America.

The aim of this study was to evaluate the response of cholinesterase as a biomarker of exposure to pesticides used in banana production as well as the suitability of *A. aeneus* as a sentinel species to detect early effects of pesticides in the Caribbean area of Costa Rica.

Material and Methods

In the present study, three methods were followed to evaluate ChE activity in *A. aeneus*: 1) Laboratory bioassays where fish were exposed to an organophosphate pesticide (ethoprophos); 2) exposure of caged fish placed at sites in a contamination gradient downstream from banana plantations; and 3) estimation of ChE activity measurements in fish captured at sites with different degree of pesticide exposure.

Fish collection and sampling area : All sites used in this study are located in the Caribbean zone of Costa Rica (Fig 1). Fish were captured using a cast net. At all sites where fish were collected or exposed, water temperature, salinity and dissolved oxygen (DO) were measured and surface water samples were also collected for chemical analyses. The water samples were collected in acetone-washed 5 l glass jars and transported on ice to the chemistry laboratory at IRET for pesticide residue analysis. Fish used as bio assays were transported alive in aerated containers to the laboratory. Fish for the caging experiments were transported alive in aerated containers to other sites where they were placed out in cages (see below). Fish for ChE estimation and pesticide residue analyses were killed on site immediately after capture and transported on ice to the laboratory and kept frozen (-20 °C) until analysis.

Fish assay in the laboratory : In order to test if fish that were chronically exposed to pesticides had acquired some tolerance to

these pollutants, laboratory exposure assay was carried out with fish collected from an assumed polluted site and also with fish from less exposed site. Site Canal Batán (CB), located close to banana plantation, with fish assumed to be frequently exposed to pesticides. Río Santa Clara site (RSC), which is located upstream to most of the plantations was assumed to be less exposed to pesticides.

In laboratory, fish were exposed to control, 0.25, 0.5 and 1.0 mg l^{-1} of ethoprophos. A stock solution of ethoprophos (20 mg l^{-1}) was prepared by dissolving concentrated 98.1% of active ingredient (Dr. Ehrenstorfer GmbH, Germany) in ultrapure water (MILLIPORE). For each treatment, ten fish were placed in a 60 l aquarium with filtered (MILLIPORE) and UV-treated (PURA), aerated water. Fish were acclimatized for 48 hr before exposure and fed once a day. After acclimatization, 20% of water in the tanks was replaced.Immediately, aliquots of the ethoprophos stock solution were added to the tanks and fish were exposed for 96 hr. During exposure, tanks were constantly aerated and no food was provided; water temperature varied between 20.5 and 23.5°C, and a natural photoperiod was maintained. Eventual dying fish were immediately removed from the tank and frozen at -20 °C. At the end of the assay, remaining living fish were collected and frozen until dissection.

***In situ* exposure :** Fish captured at RSC were transported in aerated containers filled with water from the site and then exposed at three different sites, representing a gradient along the Madre de Dios River, from 1) high exposure to pesticides at a site called "Canal Sahara" (CS) immediately downstream of the plantations; 2) moderate exposure at a site called "Goshen" (G) i.e. a bit further downstream in the river Rio Madre de Dios; and 3) a site in the Madre de Dios lagoon (LMD), a coastal lagoon that also receives seawater from the Caribbean Sea, assumed to have a lower exposure. Site RSC was used as control.

For exposure, five fish were placed in a cage (size: 45 x 25 x 25 cm; mesh size: 0.5 cm) and two replicate cages were placed at each site. After 24 hr, cages were carefully hauled up to the surface to check that the fish were alive and to analyze physico-

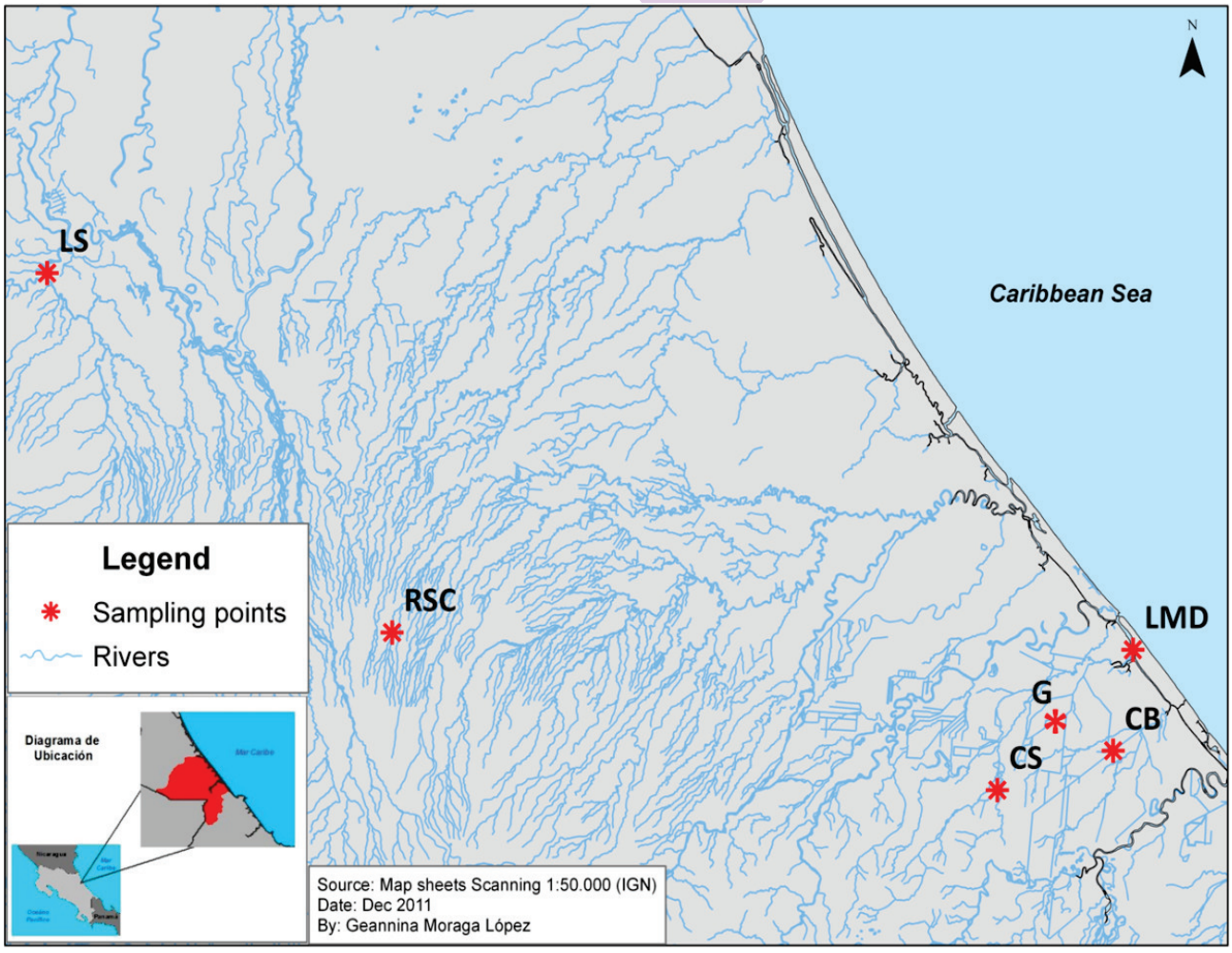

Fig. 1 : Location of sites studied in the Caribbean zone of Costa Rica

chemical parameters of the water. After 48 hr, the cages were hauled back up again and the fish were frozen on liquid nitrogen, transported on ice to the laboratory and kept frozen at -20°C until dissection and analyses. Surface water samples were collected at all sites for chemical analysis of pesticide residues.

ChE determination in fish captured at sites with different degree of pesticide exposure: For determination of ChE activity in fish directly collected from La Selva (LS), a control site, Canal Batán (CB), and Laguna Madre de Dios (LMD). Thirty fish were collected per site.

Tissue preparation : Frozen fish were thawed, weighed and measured before dissection. Then, fish were decapitated, cranium was opened and whole brain was extracted. Also, a muscle sample of about 100 mg was obtained from the dorsal myotome, excluding scales, skin and spines, put in a microtube and immediately frozen at -20°C until homogenization. An additional muscle sample was taken from fish of the 96 hr static assay with fish from RSC, for chemical analysis of pesticide residues.

Brain and muscle samples were homogenized in 1 ml of cold 100 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.2) using an ultrasonic homogenizer (Branson Sonifier 450, Dambury, USA). Brain samples were then centrifuged at 8160 g for 3 min and muscle samples were centrifuged for 6 min at same speed. Supernatants were transferred to new microtubes and diluted with phosphate buffer (brain 1:3 and muscle 1:6) for enzyme analysis.

Protein estimation and enzyme assays : Protein content in both brain and muscle samples was determined following the method of Bradford (1976) adapted to a microplate reader. Bovine -globulin (Bio-Rad) was used as standard. ChE activity was measured by the method of Ellman *et al.* (1961), adapted to microplate by Guihermino *et al.* (1996). No distinction was done between AChE and BuChE and the activity measured was expressed as total ChE. The Ellman assay was carried out by exposing the samples to a reaction solution (1 mM Acetylthio choline and 0.1 mM DTNB). Absorbance was measured at 412 nm in a micro plate reader (Multi Skan MS, type 352, Serial 35200, Labsystems, Helsinki, Finland) after 10 and 15 min the reaction was initiated. ChE activity was expressed as U mg^{-1}, whrere U = $nmol*min^{-1}$.

Analysis of pesticide residue : Extraction of water samples by solid phase extraction (SPE) and analysis of pesticide residues were done using gas chromatography with electron capture detection (GC-ECD) for detection of non-polar pesticides and high performance liquid chromatography (HPLC) for polar pesticides. An electro capture detector (ECD, Shimadzu GC17 or Fisons Mega 2) or flame photometer detector (FPD, Varian 3500) and a photodiode array detector (PDA, LC10 and SPD-M10A, Shimadzu) were used for GC and HPLC analysis, respectively. Muscle samples from fish used at 96 hr test were pooled by concentration and homogenized using an ultraturrax homogenizer (Ultra-Turrax T8, IKA Labortechnik). Then, 25 g of homogenate was extracted with 30 ml ether, 30 ml dichloro methane and 30 ml acetone. Thereafter samples were analyzed for ethoprophos residues with GC-FPD.

Data analysis: LC_{50} values were calculated by a regression of probit transformed response (mortality) data against log10 transformed treatments, using SPSS 17.0 software (SPSS Statistics for Windows, Version 17.0. Chicago: SPSS Inc). Differences between ChE activity in fish from the 96-hour assay were tested using one- and two way ANOVA followed by Student-Newman-Keuls post-hoc tests (SNK). In all cases, fish were analyzed for ChE activily individually and differences between treatments were considered significant at a probability level of p 0.05. Figures are presented as mean values with respective standard deviations.

Results and Discussion

In the present study, ChE activity in *A. aeneus* was evaluated as a biomarker in three exposure scenarios: by exposing fish *in vitro* to ethoprophos; by capturing fish at a control site and exposing them in sites representing a gradient of pesticide exposure (*in situ* exposure); and by measuring ChE activity in fish captured at sites with assumed different degrees of exposure to pesticides.

Table 1 summarizes the physico-chemical properties of water recorded at the sites of fish sampling and of in situ exposures. Table 2 presents the pesticide residues detected at the sites of the study. As it shows, no organophosphate or carbamate pesticides were detected at any of the sites. However, presence of other pesticides was detected in three of them.

Fish collected from CB for *in vitro* tests were of both sexes with average length of 52.2 + 4.4 mm and weight of 3.1 + 0.9 g. Fish collected from RSC were of both sexes with average length of 70.1 + 4.8 mm and weight of 7.2 + 1.5 g. The 96h LC_{50} of ethoprophos for *A. aeneus* captured at RSC ranged from 0.25 to 0.5 mg l^{-1}. Meanwhile, LC_{50} calculated in fish collected from CB was slightly higher (0.54 mg l^{-1}).

ChE activity in brain and muscle of fish exposed to ethoprophos in the laboratory was significantly lower as compared to control, even at lowest concentration of ethoprophos tested, inhibition in ChE activity was observed. This was the case in fish collected both from CB and from RSC (Fig. 2 and 3). ChE inhibition in brain of fish exposed to ethoprophos was gradual with increasing concentrations of pesticide (Fig. 2). In muscle, a similar ChE inhibition was observed among fish exposed to different concentrations of pesticides (Fig. 3). Comparing the degree of ChE inhibition, with respect to control fish, due to

ethoprophos treatment, no significant difference was observed between sites. Nevertheless, fish collected from CB showed a higher ChE activity ($p<0.05$) in brains compared to fish from RSC. This was observed in all ethoprophos treatment as well as in control (Fig. 2). The difference in ChE activity between sites was not observed in muscle ($p>0.05$), this in turn may be due to the fact that muscle contains less nerve tissue and less acetyl choline than brain tissue and changes in AChE activity are thus more difficult to detect in muscle than in brain (Fig. 3).

Quantification of ethoprophos in the muscles of fish exposed for 96 hours to ethoprophos showed the presence of the pesticide in tissue in increasing concentration according to treatments (Table 3).

Table 1 : Physico-chemical properties of water collected from different study sites

Site	DO (mg l^{-1})	pH	Temperature (°C)	Conductivity (µS cm^{-1})
LMD	4.9	6.9	28.1	710
LS	6.25	6.7	25.4	306
RSC	8.2	7.0	23.2	54.9
CB	5.9	7.0	28.5	372
CS	6.9	7.2	28.2	154
CG	6.1	6.3	26.7	180

Table 2 : Pesticides detected at study site

Site	Pesticide detected	Amount
LMD	Difenoconazole[b]	<0.1 µg l^{-1}
	Bromacil[c]	0.3 µg l^{-1}
LS	nd	-
RSC	nd	-
CB	Propiconazole[c]	<0.1 µg l^{-1}
	Difenoconazole[a]	1 µg l^{-1}
CS	nd	-
CG	Difenoconazole[b]	0.1 µg l^{-1}

nd= no pesticides detected; [a] = detected in site when fish were captured for *in vitro* tests; [b] = detected in site when fish were exposed in cages; [c] = detected in site when fish were captured for direct ChE analysis

Table 3 : Residues of ethoprophos in muscle of *A. aeneus* after 96 hr of exposure

Concentration of ethoprophos in water (mg l^{-1})	Concentration of ethoprophos in muscle sample (µg g^{-1} wet weight)
0	nd
0.25	0.6
0.5	1.9
1	3.2

nd= not detected

Fish used for caging experiment were of both sexes with average length of 81.3 +11.16 mm and weight of 12.5 + 4.9 g. After 48 hr of exposure, no mortalities occurred at any of the sites. Most fish, however, had damaged fins, indicating severe stress and aggressive behavior in the cages. No significant differences in ChE activity were observed between the sites neither in muscle nor in brain tissue (Fig. 4).

Fish collected from La Selva, LMD and CB were of both sexes. Their average length was 63.7±11.9 mm; 64.8±6.4 mm and 58.6±4.1 mm, respectively.

Brain ChE activity analyzed in field fish revealed no significant difference between any of the sites sampled (LS, CB and LMD) (Fig 5). However, in muscle, a significantly lower enzyme activity (p0.001) was observed in fish from LMD compared to fish from CB. No significant difference was established in muscle activity between CB and LMD compared to LS site.

Considering that the concentration of pesticide residues in environmental water are generally very low and often too low to be detected, the sensitivity of *A. aeneus* to ethoprophos in the laboratory assays suggests that ChE a suitable biomarker and that this species should be a good species to use in monitoring studies. Detection of pesticide residues in water samples is further complicated by the fact that the pesticides generally come in pulses with peak concentrations after their emission. Then, because of dilution and degradation, the levels detected in water can drop strongly in few days (Zhao and Hwang, 2009). Regarding the pesticide tested, ethoprophos has been reported to be persistent in water (Robinson *et al.,* 1999). It has also been described that degradation of the compound in soil is accelerated by high temperature and humidity (USEPA, 1999), which are the regular environmental conditions of the study area. In this regard, the evaluation of a biomarker as cholinesterase would give information about the exposure of the organisms to levels of the pesticide higher than those detected by chemical means because recovery of ChE activity after exposure to an organophosphate pesticide would take more time than degradation and dilution of the compound in the environment (Gruber and Munn, 1998; Wijeyaratne and Pathiratne, 2006; Van Cong *et al.,* 2008; Zhao and Hwang, 2009).

High concentration of ethoprophos was detected in fish muscle after *in vitro* exposure to the pesticide. This suggests that fish muscle would be a good matrix for detection of this pesticide as it accumulates there.

When *A. aeneus* captured from the site highly contaminated by the banana plantation (CB) were exposed to ethoprophos, their observed LC_{50} level was higher compared to fish captured at a site less exposed to pesticides (RSC). This observation suggests that chronic exposure to pesticides could

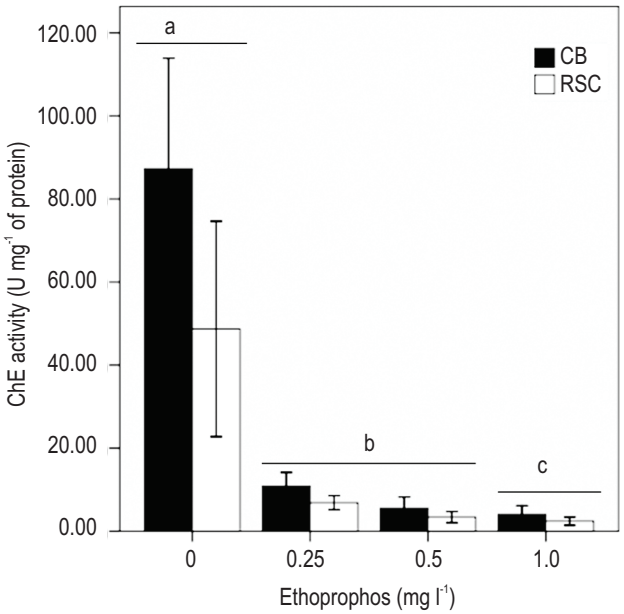

Fig. 2 : ChE activity in brain of fish collected from CB and RSC and treated with different concentrations of Ethoprophos. Significant treatment effect is shown (a > b and c; b > c. One-way and two-way ANOVA, $p < 0.05$). Error bars represent SD, horizontal bars indicate post hoc groups a, b and c (SNK, $p < 0.05$)

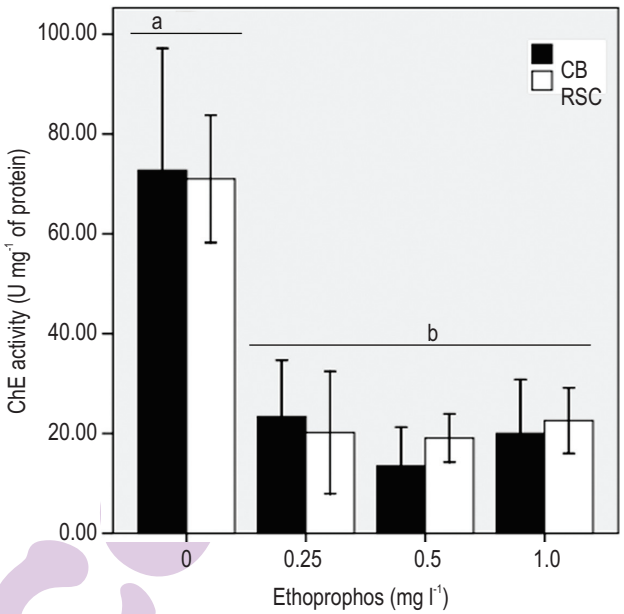

Fig. 3 : ChE activity in muscle of fish collected from CB and RSC and treated with different concentrations of Ethoprophos. Significant treatment effect is shown (a>b. One-way and two-way ANOVA, $p<0.05$). Error bars represent SD, horizontal bars indicate post hoc groups a and b (SNK, $p<0.05$)

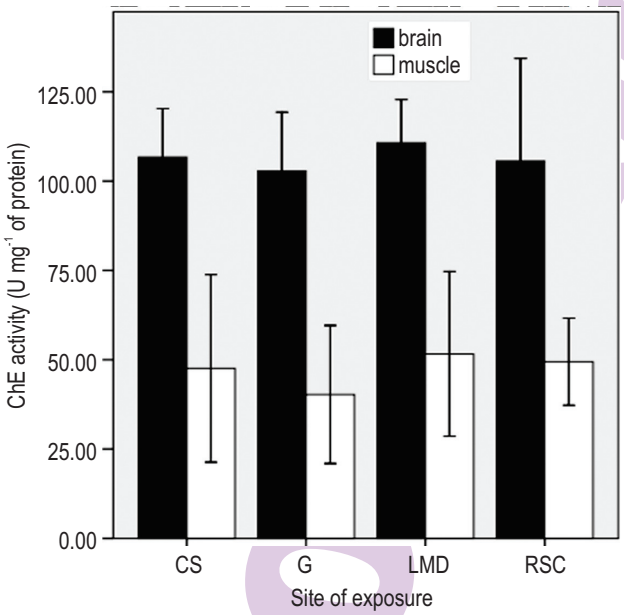

Fig. 4 : Cholinesterase activity in brain and muscle of *A. aeneus* exposed in cages at different sites. No significant differences in ChE activity was detected between sites. Error bars represent SD

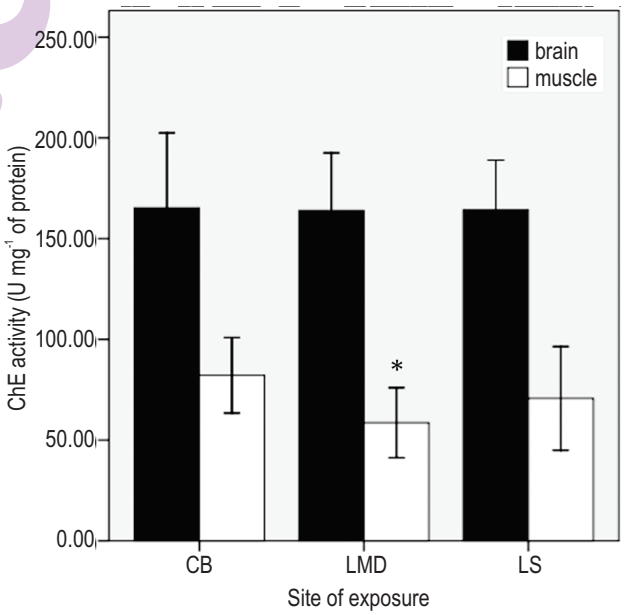

Fig. 5 : Cholinesterase activity in brain and muscle of *A. aeneus* collected from different sites. Error bars represent SD. Activity in muscle was significantly lower in LMD than in CB, $p < 0.001$

generate some tolerance on the fish population, or selection for fish that are more resistant to pollutants. Forbes (2006) demonstrated that exposure history is an important aspect to be considered in monitoring studies with biomarkers.

A higher brain ChE activity was observed in fish collected from CB compared to fish from RSC after exposure to ethoprophos treatment. Earlier it has been observed that ChE activity in *A. aeneus* muscle decreased as the size of fish

increased (Pfennig, 2006). However, this did not occur with brain ChE activity, which was the case in this study. Apart from that difference, the effect caused by exposure to ethoprophos in brains of fish from both sites was similar, confirming the accurate response of ChE as a biomarker.

In situ exposure using caging experiment did not show any difference in ChE activity between sites. This could be due to the short duration (48 hr) of exposure and that no pesticide pulses occurred during this time. As the fish were stressed and aggressive in the cages a longer exposure was not possible. *A. aeneus´s* aggressiveness suggested that less fish per cage or larger cages are necessary or that this species is not suitable for caging experiments. The observation of no differences in ChE activity between fish exposed in cages in different sites, agrees with the fact that no organophosphate or carbamate pesticides were detected in any of those sites at moments of capture or exposure. Apart from pesticides, some difference in the physico-chemical characterstics like dissolved oxygen concentration, temperature and conductivity was observed between sites. These parameters could also influence the ChE activity, e.g increased temperature was found to reduce AChE in killfish (Baslow and Negrelli, 1964). In bluegill, however, a range of temperature between 20 – 31°C did not affect ChE activity (Beauvais *et al.,* 2002). As temperature at all sites was with in range, and no difference in enzyme activity was observed, it can be inferred that ChE activity in *A. aeneus* was probably not affected at those temperatures.

A higher ChE activity in fish caught from the wild, was observed in the muscles of fish collected from Canal Batán (CB), i.e. near the plantations compared to the less exposed Laguna Madre de Dios (LMD). This may be due to difference in the size of the fish as mentioned before (Pfennig, 2006). De la Torre *et al*. (2007) reported that pollutants other than organophosphates or carbamates affected fish in LMD. Residues of bromacil were detected at LMD. Furthermore, LMD is a reservoir that collects many effluents and may cause an exposure to trace levels of several pollutants there, which could act in an additive or synergistic way. And also those pollutants might have degraded under detectable levels while the inhibition of the enzyme was still measurable. Higher ChE activity observed in brain and muscle of field fish compared to fish exposed in cages may be the evident stress that fish were submitted to in cages as well as due to differences in the size of the fish. No traces of organophosphate or carbamate pesticides were detected in any of the sites study. However, detection of other pesticides in LMD, CB and Goshen sites demonstrates the discharge of run-off from plantations in to these water bodies.

This work gave promising results regarding the use of ChE as a biomarker for pesticide pollution in tropical areas. As well, results suggest that A. aeneus would be a reliable sentinel species for the evaluation of biomarkers. More research is necessary to test sensitivity of this species to other pesticides and the responses of ChE and other biomarkers, in order to use this kind of information as early warning systems and weight of evidence of pesticide exposure in ecological risk assessment procedures.

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