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Inhibition of acetylcholinesterase activities in whitegoby, *Glossogobius giuris* from the East Bay of Laguna Lake, Philippines

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Abstract Laguna Lake, the largest freshwater lake in the Philippines, has been threatened by fertilizers and pesticides runoff from agricultural land use in the eastern bay. *Glossogobius giuris*, whitegoby, is one of the native and commercially important fishes in the lake and is reported to be of declining population. Inhibition of acetylcholinesterase (AChE) activity is widely known as biomarker of exposure to organophosphates and carbamates pesticides. The study determined AChE activities in brain and muscle using rapid colorimetric method in wild fish populations obtained from two predominantly agricultural sites (Bay and Santa Cruz) in Laguna and a reference population, reared in concrete tanks. Measured brain AChE activity across populations was significantly ($P<0.05$) higher than muscle. This resulted to a significantly ($P<0.05$) higher inhibition rate in muscle than brain in wild populations. However, inhibition rates were not significantly ($P>0.05$) different between agricultural sites. Depressed AChE activity levels may indicate fish exposure and toxicity to anticholinesterase pesticides in the eastern bay of the lake. This could be supported by carbamates and organophosphates usage in rice and vegetable production along the lakeshore as revealed through key informant interviews and focus group discussions. Findings may assist in the monitoring of the lake's ecological status and on management actions pertaining to agricultural inputs and other wastes that possibly contribute to the deteriorating water quality and declining fish production in the lake. Further studies with increased sample size from other bays and tributaries of the lake, and the analyses of water and sediment samples for detection of pesticides and other neurotoxic compounds (e.g. heavy metals) affecting AChE activity levels are recommended for an overall assessment on the status of this fishery resource.

Keywords: biomarker, carbamate, fish muscle, organophosphate

Introduction

Laguna Lake, also known as Laguna de Bay, is classified as Class C inland water (suitable for fishery) (Department of Environment and Natural

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Resources, 1990) providing various ecosystem services like fisheries and aquaculture, navigation, hydroelectric power generation, temporary storage of floodwater, sources of irrigation and domestic water, and recreation. However, through the years, increased sedimentation from denuded uplands, contaminants from agricultural activities, domestic wastes, industrial discharges, effluents from dumpsites and intensification of fish production contributed to the ecological status of the lake (Global Footprint Network *et al*., 2013).

Pollution and contributory factors such as introduction of exotic species (janitor fish (*Pterygoplichthys sp.*) and knife fish (*Chitala ornata*)), and destructive harvesting in the lake have led to the decline in fish production (Lasco and Espaldon, 2005). The white goby, *Glossogobius giuris* (Hamilton, 1822), locally known as "biyang puti", is one of the economically important native fishes of dwindling stocks in the lake. This goby species is characterized by a fused pelvic fin at the antero-ventral portion of the body, dwells at the bottom substrate and attaches into rocks, and is widely distributed in rivers and lakes throughout the country.

Biomarkers are functional measures of exposure to various stressors (Adams *et al*., 2001) or as changes in biological response that can be related to exposure to environmental chemicals (Sanchez *et al*., 2008). These are useful tools in monitoring biotic 'health' and give an indirect measure of possible environmental pollution or degradation (Richardson *et al*., 2010; Sole *et al*., 2006; Fulton and Key, 2001; Kirby *et al*., 2000). Inhibition of acetylcholinesterase (AChE) activity has been assayed in fish species as specific biomarker of organophosphates and carbamates pesticides - even when these compounds are not present in the water (Rodr ǵuez-Fuentes *et al*., 2008; Rodr ǵuez-Fuentes and Gold-Bouchot, 2004). Organophosphorus and carbamate pesticides are widely used in tropical agriculture. Their relatively fast hydrolysis and low persistence in the environment have supported their increasing use. However, their toxicity to non-target organisms such as birds and aquatic organisms, together with the large amounts used, constitute a threat to human health and the environment (Arufe *et al*., 2007).These pesticides can persist for days in the environment but their action on tissues may last for weeks, making it more advantageous to evaluate biomarkers such as cholinesterases than chemical residues for environmental monitoring programs (Assis *et al*., 2012).

The use of pesticides is an essential part for the production of vegetables and rice in the Philippines. Steep slopes and heavy rains in the country could exacerbate the problem of off-site migration and risk of contaminating water resources (Fabro and Varca, 2012).

The study investigated acetylcholinesterase activity inhibition in *G. giuris* populations from the East Bay of Laguna Lake and a reference population. Results of the study may assist in the conservation and management programs, particularly in the monitoring of the lake's ecological status and on management actions pertaining to agricultural inputs and other wastes that possibly contribute to the deteriorating water quality and declining fish production in the lake.

Materials and methods

Study area and fish sampling

Laguna Lake (13º 55' to 14º 50' N and 120º 50' to 121º 45' E) is situated in Luzon Island and covers a watershed area of 3,820 km^2. The lake is divided into three distinct bays: the West, Central, and East. The main land use in the catchments that drain into the eastern bay of the lake is agricultural. Wild populations of sexually matured *G. giuris* were collected from selected sites in the East Bay such as Bay (14.1320 ° N, 121.2569 ° E) (*n*=15) and Santa Cruz (14.2691 °N, 121.4113 °E) (*n*=15) in the province of Laguna. On the otherhand, reference population (*n*=15) was obtained from the University of the Philippines, Limnological Research Station (UPLB-LRS), where gobies are reared in concrete tanks. Live wild fish samples were collected through coordination with fisherfolks using passive fishing gears like fyke nets and cover pots from June to October 2015.

Fish analyses

Standard length was measured from the tip of the mouth to the caudal peduncle region whereas total length was measured from the tip of the mouth to the tip of the caudal fin. Standard and total lengths were determined using a digital caliper (cm) whereas wet weight (g) was determined using a digital weighing balance. Sexes were identified based on external morphology in which females exhibited broad and yellowish papilla while males had slender, pointed and whitish papilla. Sexes were further checked with the presence of ovary for female or testes for male.

The condition factor (K) was estimated from the relationship:

$$K = \frac{W}{L3} \times 100$$

where, W is weight of fish (g) and L is length (cm).

Measurement of acetylcholinesterase activity levels

AChE was determined using the rapid colorimetric method of Ellman *et al.* (1961) and analytical grade chemicals. Brain and muscle tissues were excised and homogenized at approximately 20 mg of tissue per mL of phosphate buffer (pH 8, 0.1 M). An aliquot of 0.4 mL homogenate was added to a cuvette containing 2.6 mL of phosphate buffer (pH 8). Dithiobisnitrobenzoic acid reagent of 100 µL was then added to the cuvette. Absorbance readings were measured at 412 nm. Acetylcholine iodide of 20 µL was added as substrate. Changes in absorbance were recorded for seven minutes and were computed using the formula:

$$R = \frac{\Delta A}{1.36\,(104)} \times \frac{1}{(400/3120)\,Co} = 5.74\,(10\text{-}4)\,\frac{\Delta A}{Co}$$

where, R is rate expressed in moles substrate hydrolyzed per min. per g of tissue, ΔA is change in absorbance per min., and Co is original concentration of tissue (mg mL^{-1}).

Assessment of pesticide usage

The usage of pesticides in rice and vegetable production was assessed with the assistance of the Municipal Agriculture Offices of Santa Cruz and Bay. Key informants (farmers) were selected based on the needed data and as advised by the municipal agriculturists and technicians. In parallel, focus group discussions were conducted to farmers' associations in both study sites.

Data analyses

Means of AChE activity were analyzed for significant ($p<0.05$) differences using one-way Analysis of Variance (ANOVA) and means were compared using Tukey's test. Meanwhile, Student's t-test was used in the comparison of inhibition rates. The correlations between AChE activity in both tissues (brain and muscle) and total length, standard length and body weight of fish were analyzed by calculating Pearson product moment correlation coefficients (r).

Results

AChE activity levels and inhibition rates in brain and muscle of G. giuris

Brain and muscle AChE activities expressed in mol min^{-1} g^{-1} tissue x 10^{-2} of *G. giuris* obtained from three sites (Reference, Bay and Santa Cruz) are presented in Figure 1. AChE activity level in brain across populations or sites was significantly ($P<0.05$) higher than AChE activity in muscle. Both brain (0.0127 mol min^{-1} g^{-1}tissue) and muscle (0.0108 mol min^{-1} g^{-1} tissue) AChE activities were highest in the Reference (reared) population. These detected values were significantly different ($P<0.05$) from the wild populations (Bay and Santa Cruz), which were of comparable AChE activities for both tissues.

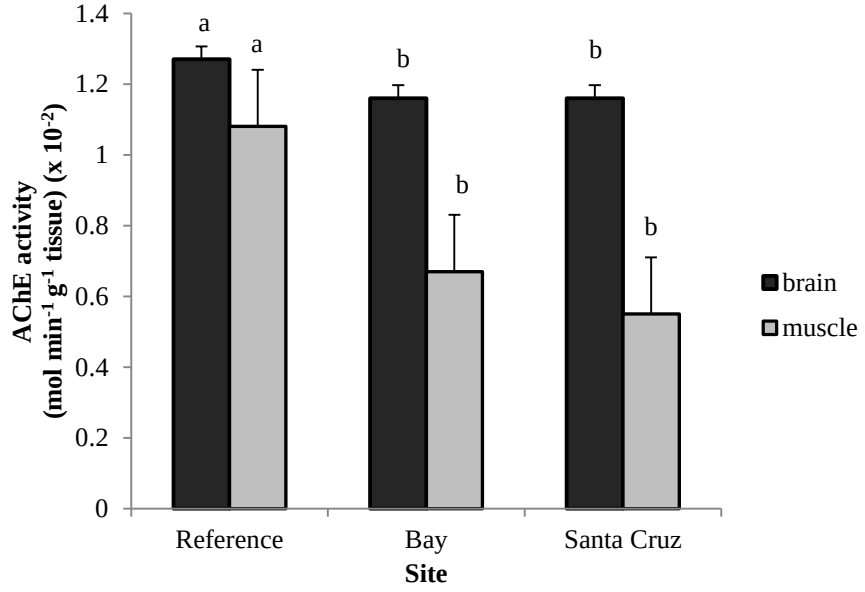

Figure 1. Brain and muscle AChE activities (mol min^{-1} g^{-1} tissue) (x 10^{-2}) of *G. giuris* populations from three sites: Reference, Bay and Santa Cruz (*n*=15 per site). Values are expressed as mean±SE. Different letters denote significant difference at 0.05 level of significance

The difference in AChE activity levels between reference and wild groups (Bay and Santa Cruz) were translated to inhibition levels. Figure 2 shows the inhibition rates of AChE activity levels in brain and muscle of *G. giuris* obtained from Bay and Santa Cruz, Laguna. Between tissues, computed percent inhibition of muscle AChE activity (38.02% Bay, 49.38% Santa Cruz)

was significantly higher (P<0.05) as compared to brain AChE activity in both sites (8.71% Bay, 8.40% Santa Cruz). However, these inhibition rates for brain and muscle AChE activity levels were not significantly different (P>0.05) between sites.

Muscle activity levels were generally of significant (P<0.05) negative correlation with total length, standard length and body weight. When compared between sexes, AChE activity levels of both *G. giuris* tissues were not significantly different (P>0.05) across populations (sites).

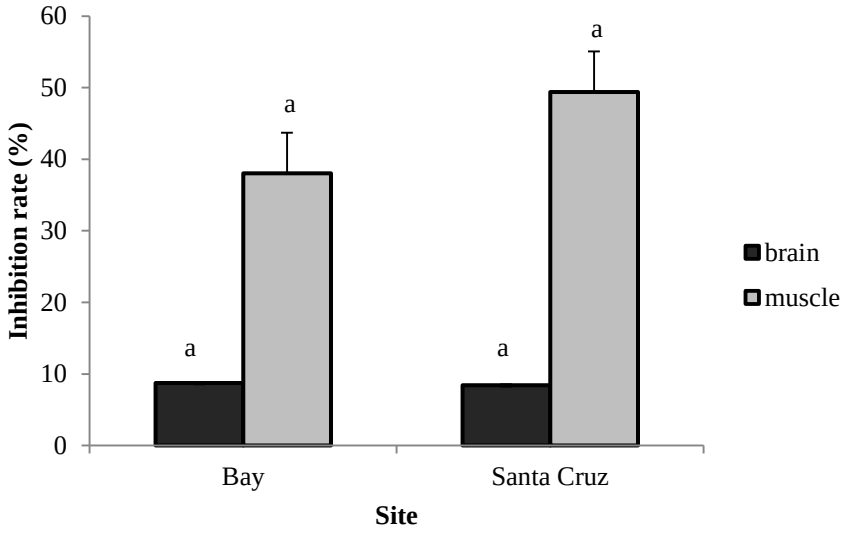

Figure 2. Percent inhibition of AChE activity in brain and muscle of *G. giuris* wild populations (*n*=15 per site). Values are expressed as mean± SE. Similar letters denote insignificant difference at 0.05 level of significance

Condition factor of G. giuris populations

Total length, standard length, condition factor and body weight of *G. giuris* populations subjected to AChE determination are shown in Table 1. Lengths and body weights of wild populations were significantly higher (P<0.05) than reference population. However, condition factor (K) of all populations computed based on total and standard lengths were not significantly different (P>0.05).

Table 1. Total length, standard length, condition factor and body weight of *G. giuris* populations

Site	Total length (cm)	Condition factor (K)	Standard length (cm)	Condition factor (K)	Body weight (g)
Reference *n*=15	11.85±1.48[b]	0.74±0.06[a]	9.33±1.18[b]	1.53±0.12[a]	12.83±5.10[b]
Bay *n*=15	15.76±2.79[a]	0.72±0.08[a]	12.32±2.19[a]	1.51±0.17[a]	31.65±20.17[a]
Santa Cruz *n*=15	16.78±2.49[a]	0.70±0.12[a]	13.09±2.32[a]	1.47±0.13[a]	35.54±16.39[a]

Values are expressed in mean± standard deviation. Superscripts with different letters across sites denote significance at 0.05 level.

Pesticide usage along east shore of Laguna Lake

Interviews with rice farmers and focus group discussions with farmers' associations from selected barangays and Municipal Agriculture Office (MAO) technicians revealed that majority of herbicides used for rice production in Bay and Santa Cruz were butachlor, bispyribac-sodium, 2,4-D (Dichlorophenoxyacetic acid) and glyphosate while the insecticide mostly used was cypermethrin (pyrethroid-based). It could be noted that more varieties of insecticides (carbofuran, lambda cyhalothrin, malathion and parathion-ethyl) were used in Santa Cruz as compared to Bay. Herbicides were applied once to twice per cropping whereas insecticides were applied once to thrice per cropping in both study sites. Average spray loading or application per hectare of both herbicides and insecticides was greater in Santa Cruz (10 and 16 tank loads) than Bay (4.5 and 5.5 tankloads). A tank load is equivalent to 16 L. Niclosamide (molluscicide) was used by rice farmers to control golden apple snails in the study sites.

On the otherhand, majority of vegetable crops grown in both sites were eggplant, lady's finger, bittergourd and chinese cabbage. Other vegetables grown in Bay include tomato, string beans, mustard greens, radish and swamp cabbage. With respect to insecticide use, chlorpyrifos, profenofos, and hydrochloride (thiocarbamate) were mostly applied for vegetable crops in both areas. Other insecticides used for vegetables were carbofuran, cypermethrin, methomyl, carbaryl and thiamethoxam. Based from interview with farmers, insecticides were more frequently used for vegetables (every other day, every five days, every week throughout the planting season depending on the crop).

Among the pesticides used by farmers in the study sites, malathion, parathion-ethyl or diethyl parathion, profenofos, chlorpyrifos and glyphosate

are classified as organophosphates. Those classified as carbamates are carbofuran, carbaryl and methomyl.

Discussion

The effects observed in the study (AChE inhibition in muscle: 38.02%, 49.38%) may indicate the presence of organophosphorous (OP) or carbamates molecules in the study sites (Bay and Santa Cruz). AChE activity inhibition above 20% has been suggested as indicative of exposure to anticholinesterase agents (Menendez-Helman *et al*., 2015). Moreover, considering the low inhibition percentages (<50%) in the study, this would predict severe neurotoxic effects in fish from the sites (Bay and Santa Cruz) since fish with less than 80% normal ChE activity are considered to have suffered from the exposure to some anti-cholinesterase substances (Flammarion *et al*., 2002; Sturm *et al*., 1999).

AChE is found in fish brain and muscle tissue controlling a large proportion of physiological and behavioural responses (Pan and Dutta, 1998; Kirby *et al*., 2000). It has been shown that AChE activity is important for physiological functions such as locomotor capacity, predator evasion, prey location, orientation towards food and feeding, spatial distribution pattern and social interactions (Bradbury *et al*., 2008). Depressed AChE activity due to exposure to a cholinesterase inhibitor may lead to slowed breakdown of released acetylcholine, resulting in overstimulation of the target cells, which can be fatal (Padilla, 1995; Sancho *et al*., 2000).

Meanwhile, percentage inhibition on brain AChE activity levels (8.71% Bay, 8.40% Santa Cruz) obtained were lower than the suggested threshold (~20% inhibition rate) by the United States Environmental Protection Agency (EPA) on diagnosis of organophosphate poisoning (US EPA, 1999 as cited by Ugaddan and Ocampo, 2014). Development of tolerance may have been a result of several underlying factors governing the survival of fishes. Brain enzyme inhibition did not rise up (8.71% Bay, 8.40% Santa Cruz), suggesting that fish can still have some mechanisms of controlling its bodily activity, eventhough inhibition rate in muscle was high (38.02% Bay, 49.38% Santa Cruz). A decrease in number of receptors to compensate for the toxicity is one probable mechanism for their tolerance. The study of Fitzgerald and Costa (1993) as cited by Ugaddan and Ocampo (2014), using rat as a vertebrate model, was proposed that development of tolerance to OP toxicity is related to decrease in cholinergic muscarinic receptor density in brain as well as peripheral tissues. Tolerance may also be related to a decrease in nicotinic

binding sites along neuromuscular junction (Gupta *et al.*, 1985 as cited by Ugaddan and Ocampo, 2014).

US EPA (1999) suggested that 50% inhibition or more in AChE activity can be considered a good diagnostic threshold for determining cause of death. However, in some species, fish survival has been observed even in brain AChE activity inhibition of 90–95% (Sturm *et al*., 2000; Fulton and Key, 2001; Ferrari *et al*., 2004).

Condition factor (K) shows the degree of well-being of fish in their habitat and is expressed by 'coefficient of condition' also known as length – weight factor. It is a measure of various ecological and biological factors such as degree of fitness, gonad development and the suitability of the environment with regards to the feeding condition (Nehemia *et al*., 2012). Computed K in the study were higher than 1 indicating better condition of fish. Yildirim *et al*. (2014) reported that decreased K values may be more dependent on the availability of nutrients in the water than on the water pollution status or other stress factors.

Results of the present study support previous studies that revealed inverse relationship between enzymatic (AChE) activity and fish size (Menendez-Helman *et al*., 2015; Chandrasekara and Pathiratne, 2007; Beauvais *et al*., 2002; Flammarion *et al*., 2002). Bigger fishes have larger total body surface area ratio than smaller ones, thus allowing them to assimilate and even detoxify more substances at a faster rate, making them tolerant to a given dose of toxicant (Ugaddan and Ocampo, 2014).

Moreover, findings of the present study agreed to previous reports showing that the variability of AChE activities is not related to sex or reproduction period in most fishes (Beauvais *et al*., 2002; Flammarion *et al*., 2002). Correlation analysis showed no strong links of AChE activity with fish sex and gonado-somatic index in flounder (*Platichthys flesus*) populations (Kirby *et al*., 2000).

Inhibition of AChE activity levels, as sensitive biomarker of organosphosphates and carbamates exposure, in both brain and muscle tissues of *G. giuris* could be an indication of the presence of anticholinesterase pesticides in the lake water. Pesticides may enter water bodies as a result of spray drift, leaching from the soil, surface runoff (edge-of-field runoff) during precipitation in concentrations which may exert adverse effects on non-target organisms inhabiting the area (Hamers *et al*., 2001). The inhibition in AChE activity of *G. giuris* in the study can be further supported by the existing agricultural practices, particularly on pesticide usage, in the study sites based on key informant interviews and focus group discussions. Out of the pesticides used by farmers, organophosphates include malathion, parathion-ethyl or

diethyl parathion, profenofos, chlorpyrifos and glyphosate while carbamates are carbofuran, carbaryl and methomyl. Chlorpyrifos, profenofos, and hydrochloride (thiocarbamate) were mostly applied for vegetable crops in both areas. Other insecticides used for vegetables were carbofuran, cypermethrin, methomyl, carbaryl and thiamethoxam. Insecticides were more frequently used for vegetables (every other day, every five days, every week depending on the crop).

Significantly higher AChE inhibition in fish muscle may imply potential risk in humans as *G. giuris* is an important fishery resource for human consumption. Stage of fish or fish size is significant to consider as this has significant correlation to AChE activities as supported by previous studies. The analysis of water and sediment samples for detection of pesticides and other neurotoxic compounds (e.g. heavy metals) affecting AChE activity levels should be also considered. For future research directions, increasing the sample sizes and the investigation of *G. giuris* populations from the other bays (West, Central, South) and tributaries surrounding Laguna Lake are recommended in order to have an overall assessment on the status of this fishery resource.

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