Acute toxicity of a deltamethrin based pesticide (DBP) to the Neotropical electric fish *Microsternarchus cf. bilineatus* (Gymnotiformes)

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**ABSTRACT**

Deltamethrin is a pyrethroid insecticide widely used in pest control in Brazilian agriculture. The intensive and disordered use of this pesticide in the Amazon region can drive it into aquatic ecosystems in several ways, but mainly by runoff and leaching. The present study was conceived to determine the acute toxicity (LC₅₀) of a deltamethrin based pesticide (DBP) and to characterize its effects on two biochemical biomarkers, glutathione-S-transferase (GST) and acetylcholinesterase (AChE), in tissues of the electric fish *Microsternarchus cf. bilineatus*. Fishes were exposed to concentrations of 1, 2, 3, 4 and 5 μg L⁻¹ of DBP for up to 96 hours. For each treatment, the absolute activity of the enzymes GST (muscle and liver) and AChE (muscle and nervous tissue) were analyzed. The LC₅₀-96 h for *Microsternarchus cf. bilineatus* was 2.15 μg L⁻¹, the lowest concentration registered for an Amazonian fish species so far. None of the concentrations tested of this insecticide affected AChE activity for the exposure period tested. A significant increase in muscle GST activity was detected only for concentrations of 2 and 3 μg L⁻¹.

**KEYWORDS:** pyrethroid insecticide, biomarkers, median LC₅₀, glutathione-S-transferase, acetylcholinesterase
INTRODUCTION

Deltamethrin is a synthetic type II pyrethroid insecticide that acts on the central nervous system of its target organisms. It is considered very toxic to fishes due to its lipophilic character, allowing a high absorption rate through the gills (Santos et al. 2007). Among the eighteen pesticides used by farmers in the metropolitan region of Manaus, Amazonas State, Brazil, the deltamethrin insecticide was the most recurrent (Waichman et al. 2002, 2007).

The acute toxicity of deltamethrin was evaluated for several Amazonian fish species (Moraes et al. 2013; Souza et al. 2020), with LC50-96h values ranging from 4 to 215 μg L−1, representing the lowest LC50 values among all tested pesticides and confirming the high toxicity of this pyrethroid to Amazonian fish. Biochemical and physiological effects of deltamethrin and other pesticides largely used in Brazil, such as glyphosate, have also been reported for other teleost species, particularly regarding their effect on the activity of glutathione S-transferase (GST) and acetylcholinesterase (AChE) (Bálint et al. 2007; Pimpao et al. 2007; Tu et al. 2012; Rossi 2013; Braz-Mota 2015; Elia et al. 2017).

GST is a phase II biotransformation enzyme that is extremely important in the detoxification mechanism of fishes, as it catalyzes the conjugation of reduced glutathione to xenobiotics or metabolites produced in phase I (Giulio and Hinton 2008). The activity of AChE has been widely used to evaluate the neurotoxic effects of pollutants (Bálint et al. 2007; Tu et al. 2012; Rossi 2013; Braz-Mota 2015). Its inhibition generates an accumulation of acetylcholine that results in a continuous and disordered transmission of nerve impulses (Soreq and Seidman 2001).

South American electric fish (order Gymnotiformes) have shown potential for use as model organisms for bioassays, with focus on the effect of different pollutants on the electric organ discharge (EOD) (Ferreira 2009; Moraes et al. 2013; Ferreira et al. 2015; Ferreira 2016; Nunes 2016). However, no data exist on the acute toxicity of any pesticide for a gymnotiform species. Gymnotiforms are present in almost all types of aquatic habitats in the Amazon and may represent up to 90% of the benthic fauna in the Amazon and Orinoco river basins (Hagedorn 1986; Alves-Gomes 1997; Marrero and Taphorn 1991). Among their most remarkable features is the electrogenic and electrosensory system (EES), which makes this group very sensitive to pollutants in their environment (Hopkins 1974; Bullock et al. 1979; Alves-Gomes 2001; Ferreira 2009; Moraes et al. 2013; Ferreira et al. 2015; Ferreira 2016; Nunes 2016). Although GST and AChE have been widely used as molecular biomarkers in studies on environmental pollution (Bálint et al. 2007; Pimpao et al. 2007; Tu et al. 2012; Rossi 2013; Braz-Mota 2015; Elia et al. 2017), these enzymes have not yet been evaluated in Amazonian electric knifefish exposed to any pollutant.

For this study, the gymnotiform Microsternarchus cf. bilineatus was chosen due to its abundance in Amazonian streams, including the metropolitan region of Manaus, where it is exposed to the increasing use of pesticides. We evaluated the acute toxicity of a deltamethrin-based pesticide (DBP) by determining the median lethal concentration (LC50-96h) and characterizing its effects on GST and AChE activity in muscle, liver and the nervous system of Microsternarchus cf. bilineatus.

MATERIAL AND METHODS

Animal capture and maintenance

Fishes were captured in September 2016 in a stream, Igarapé Tapuru (02°43′8.1″S; 60°57′39.5″W), an affluent of the Negro River, near the municipality of Novo Airão, Amazonas State, at about 180 km from the city of Manaus. Tapuru is a typical Amazonian terra firme forest stream of second to third order, with a relatively stable range of physicochemical properties throughout the year and a slightly acidic blackwater (pH 4.5), low electrical conductivity (< 30 μS cm−1) and an average annual temperature fluctuating between 25 and 27°C (J. Alves-Gomes, unpublished data). We collected 95 individuals of Microsternarchus cf. bilineatus weighing 0.89 ± 0.33 g; and measuring 6.79 ± 0.90 cm (mean ± SD). This range of body lengths corresponds to adults and sub-adults, according to the sizes of mature individuals for species of this genus recorded in this region (J. Alves-Gomes, unpublished data). Capture of the animals was authorized by Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) under license # 55408-2, whereas the experimental procedures were performed according to the protocol approved by the Ethics Committee on Animal Reserach of Instituto Nacional de Pesquisas da Amazônia (CEUA/INPA protocol # 020/2016).

We decided to adopt a conservative taxonomic treatment for the focal species Microsternarchus cf. bilineatus, due to recent findings regarding the taxonomy and systematics of the genus. For instance, molecular data has demonstrated that the previously monotypic Microsternarchus bilineatus is, in fact, a species complex, with at least five undescribed species in the Negro River basin alone (Maia and Alves-Gomes 2012). The original M. bilineatus, described from a specimen collected in Venezuela, is no longer available in its original fish collection (Cox-Fernandes and Williston 2017) and no tissues or DNA samples are currently available for the type specimen or paratypes. Furthermore, only external morphological traits are not sufficient to discriminate between the different undescribed species found in Brazil. Therefore, until DNA becomes available and/or further systematic studies clarify...
if one of the species found in the Negro River basin is *M. bilineatus*, we opted to consider it as a species to be confirmed (*Microsternarchus cf. bilineatus*). In addition, we expect no significant differences in the physiological and biochemical responses at the intrageneric level for *Microsternarchus*.

After capture, fishes were transported to the Laboratory of Behavioral Physiology and Evolution (LFCE) at INPA, in Manaus, where they were divided into groups of approximately 15 individuals and acclimatized in 40-L glass aquaria with constant filtration and aeration. The maintenance water (Na\(^+\): 1.68 mg L\(^{-1}\); K\(^+\): 0.41 mg L\(^{-1}\); Ca\(^{2+}\): 0.07 mg L\(^{-1}\); pH: 6.84; dissolved oxygen: 5.92 mg L\(^{-1}\); conductivity: 20.2 \(\mu\)S cm\(^{-1}\), and temperature: 28 °C) was obtained from an artesian well on site and kept in a 1,000-L tank with biological filtration and constant aeration before use in the aquaria. Fishes were kept in these conditions for at least seven days before beginning the experiments, and were fed daily with *Artemia salina* nauplii and *Enchytraeus albidus*. Daily, food leftovers and waste were vacuumed from the bottom of the aquaria and water was completed to the initial level.

**Deltamethrin based pesticide (DBP)**

The DBP was purchased from the commercial formulation Decis® EC (Bayer Vapi PVT LTD, Gujarat, India) with a concentration of 25 g L\(^{-1}\) of the insecticide. From the initial concentration, a fresh stock solution was prepared with distilled water to achieve a concentration of 25 mg L\(^{-1}\). The DBP’s tested concentrations were defined from the LC\(_{50}\) concentration range obtained for other Amazonian fish species in similar studies (Moraes *et al.* 2013; Rossi 2013; Souza *et al.* 2020).

**Acute toxicity of DBP**

The median lethal concentration at 96 h of exposure (LC\(_{50}\) 96 h of DBP) was determined according to the OECD protocol (OECD 1992). The test was performed using a semi-static system with total water renewal every 24 hours. Feeding was suspended 24 hours before the beginning of the test. After the acclimatization period, fishes were transferred to experimental units containing 2 L of water and increasing concentrations of DBP and a control containing no DBP. Each unit contained seven fishes, and two replicates (2-L units) were used for each concentration, so that 14 fishes were used per treatment. The physical and chemical parameters of water (temperature, electric conductivity, pH, dissolved oxygen, Na\(^+\), K\(^+\) and Ca\(^{2+}\)) were measured daily and no significant changes were observed over the experimental period (Table 1). Fishes that died during the experiment were measured, weighed, and immediately frozen in liquid nitrogen. Surviving individuals at the end of the experiment were exposed to hypothermia and euthanized by medullary section. The fish were frozen and later thawed for dissection. Samples of muscle, liver and brain tissue were excised and stored in a freezer at -80 °C (Sanyo, Japan) for later enzymatic analysis.

**Glutathione-S-transferase (GST; EC 2.5.1.18) assay**

GST activity in liver and muscle tissue was measured by a modification of the method of Keen *et al.* (1976). The tissue samples were homogenized in phosphate buffer (100 mM; pH 7.5). The homogenates were centrifuged for 5 minutes at 10,000x g and the supernatant was used to estimate enzyme activity. Briefly, 30 \(\mu\)L of the sample was added in 120 \(\mu\)L of assay buffer containing 1 mM reduced glutathione (GSH). The 1-chloro-2, 4-dinitrobenzene (CDNB, 1 mM) was used as substrate. The change in absorbance was recorded at 340 nm, and enzymatic activity was calculated using a molar extinction coefficient of 9.6 mM\(^{-1}\) cm\(^{-1}\). The enzyme activity was expressed in nmoles mg protein\(^{-1}\) min\(^{-1}\). The protein concentrations of crude extracts were estimated by the Bradford method (Bradford 1976).

**Acetylcholinesterase (AChE; EC 3.1.1.7) assay**

The enzymatic activity of AChE in brain and muscle tissue was determined with few modifications from the original method described by Ellman *et al.* (1961), using acetylthiocholine iodide (AtCh) as substrate. The tissue samples were homogenized in phosphate buffer (100 mM; pH 7.5), centrifuged for 5 minutes at 10,000x g and the supernatant was used to estimate the enzyme activity. Thus, 5 \(\mu\)L of sample (homogenized) was used in a microplate containing 120 \(\mu\)L of assay buffer. All assays were run under supersaturated substrate concentration (AtCh, 1 mM). The change in absorbance was recorded at 412 nm and the

| Parameter        | Control | 1 µg L\(^{-1}\) | 2 µg L\(^{-1}\) | 3 µg L\(^{-1}\) | 4 µg L\(^{-1}\) | 5 µg L\(^{-1}\) |
|------------------|---------|----------------|----------------|----------------|----------------|----------------|
| Temperature (°C) | 27.8 ± 0.9 | 27.7 ± 1.0 | 27.7 ± 1.1 | 27.6 ± 1.1 | 27.9 ± 0.9 | 27.6 ± 0.8 |
| Conductivity (\(\mu\)S cm\(^{-1}\)) | 160 ± 4.5 | 150 ± 2.1 | 150 ± 3.7 | 140 ± 2.7 | 150 ± 4.4 | 130 ± 3.5 |
| pH (units)       | 6.9 ± 0.5 | 7.1 ± 0.4 | 7.1 ± 0.5 | 7.0 ± 0.5 | 7.1 ± 0.5 | 7.0 ± 0.5 |
| Oxygen (mg L\(^{-1}\)) | 6.3 ± 0.4 | 5.9 ± 0.4 | 5.9 ± 0.8 | 5.4 ± 0.4 | 5.8 ± 0.9 | 5.9 ± 0.6 |
| Na\(^+\) (mg L\(^{-1}\)) | 1.63 ± 0.7 | 1.49 ± 0.3 | 1.67 ± 0.5 | 1.65 ± 0.5 | 2.04 ± 1.0 | 1.64 ± 0.4 |
| K\(^+\) (mg L\(^{-1}\)) | 0.39 ± 0.5 | 0.37 ± 0.5 | 0.40 ± 0.5 | 0.34 ± 0.5 | 0.61 ± 0.5 | 0.44 ± 0.5 |
| Ca\(^{2+}\) (mg L\(^{-1}\)) | 0.10 ± 0.1 | 0.06 ± 0.1 | 0.04 ± 0.1 | 0.06 ± 0.1 | 0.14 ± 0.1 | 0.08 ± 0.2 |
enzymatic activity was calculated using a molar extinction coefficient of 13.6 mM$^{-1}$cm$^{-1}$ (Ellman et al. 1961). The enzyme activity was expressed in nmoles mg protein$^{-1}$ min$^{-1}$.

Data analysis

The LC$_{50}$ (at 24, 48, 72 and 96 hours) was calculated with the D-Response Curve (DRR) package from the R program using a logistic model (Ritz et al. 2015). The data for GST and AChE were not normally distributed, so a Kruskal-Wallis analysis was used, followed by Dunn’s post-hoc test, which compares all treatment groups with the control. In all performed tests, the accepted significance level was 5%.

RESULTS

Overall, 42 deaths were recorded throughout the trial (Table 2), of which 38 were processed for enzymatic assays immediately after death. The other four individuals were processed between two and five hours after death (one from the 5 μg L$^{-1}$ treatment, two for the 3 μg L$^{-1}$ and one for the 4 μg L$^{-1}$).

Acute toxicity of DBP

Throughout the 96 hours of the test, behavioral changes such as rapid and circular swimming, spasms, permanence on the water surface and increased opercular activity were observed in all groups, except the control group, in which all fishes survived and no behavioral changes were observed. The first death occurred after seven hours in the 5-μg L$^{-1}$ group, and five fish (71%) were dead within the first twelve hours. The estimated LC$_{50}$-96h was 2.15 μg L$^{-1}$ with a 95% confidence interval of 1.62–2.67 μg L$^{-1}$. For the time intervals of 24, 48 and 72 h, the estimated LC$_{50}$ values were 6.12 μg L$^{-1}$, 3.21 μg L$^{-1}$, and 2.14 μg L$^{-1}$, respectively (Figure 1).

GST and AChE assays

GST activity in muscle increased for all concentrations tested relative to the control, but was significantly higher only for the concentrations of 2 and 3 μg L$^{-1}$, with average increases of 1.8 and 1.5 times over the control, respectively (Figure 2). There were no significant differences in AChE activity in muscle and nervous tissue, neither in the GST activity in liver (Table 3).

DISCUSSION

The behavioral responses observed in our fishes exposed to DBP were similar to changes that type II pyrethroid insecticides caused in fishes, such as rapid and circular swimming, which can be related to a direct damage to the nervous system (Glickman et al. 1982; Bradbury et al. 1987; Rice et al. 1997). These changes are commonly reported in pyrethroid-exposed animals and include tremors, loss of balance, and lethargy (Werner and Moran 2008). Our results indicate that DBP is highly toxic to Microsternarchus cf. bilineatus. Based on the LC$_{50}$-96h value of 2.15 μg L$^{-1}$, the electric knifefish appears to be even more sensitive to DBP than other Amazonian fish species examined to date. The median values of LC$_{50}$-96h for deltamethrin in Carnegiella strigata, Colossoma macropomum, Corydoras schwartzi, Hemigrammus rhodostomus, and Paracheirodon axelrodi ranged from 6.69 to 183.51 μg L$^{-1}$ (Souza et al. 2020). Considering these values, the lethality of deltamethrin appears to be 87 times more toxic in Microsternarchus cf. bilineatus than in Corydoras schwartzi.
an Amazonian siluriform catfish. The high sensitivity of Microsternarchus cf. bilineatus to DBP is comparable to that found for Brycon amazonicus, for which a CL₅₀ 96h of 2.6 μg L⁻¹ was estimated (Moraes et al. 2013). In general, the response of fish to pollutants is influenced by factors such as commercial product formulation and molecule stereochemistry, as well as characteristics of the species such as body size, surface/volume ratio, feeding behavior and stage of development (Moraes et al. 2013; Haverinen and Vonanen 2016). Species of Microsternarchus have incomplete squamation on the first third of the body, above the lateral line (Cox-Fernandes and Williston 2017), and such direct exposure of the skin to the water may facilitate a higher absorption of pollutants, leading to a higher sensitivity relative to other species.

The increase in muscle GST activity observed in Microsternarchus cf. bilineatus exposed to DBP was similar to the results found in other studies that tested the effect of insecticides as contaminants (Rao 2006; Monteiro et al. 2006; Maduenho and Martinez 2008; Dong et al. 2013). This seems to confirm a specific response to detoxification for DBP, suggesting that muscle GST can play an important role in pollutant elimination from the tissues. Considering that the electric organs of Microsternarchus, and gymnotiforms in general, are embryologically derived from muscle tissue (Kirschbaum and Schwassmann 2008), GST could also provide some sort of specific protection for the electrogenic tissue. An alternative, non-excludent protective action of GST may be linked to its role against products generated by oxidative stress events (Hayes et al. 2005). Many studies have noted the effectiveness of hepatic GST in detoxifying xenobiotics, and the liver is the main source of GST in fishes (Giulio and Hinton 2008). For example, Simonato et al. (2006) reported an increase in hepatic GST activity of Prochilodus lineatus exposed to diesel oil. Braz-Mota et al. (2015) detected a decrease in the activity of hepatic GST of Colossoma macropomum exposed to a glyphosate-based herbicide. However, in M. cf. bilineatus the defense mechanisms to GST were detected only in muscle tissue. It can be implied that the GST mechanism of action can occur either by the conjugation of GSH to the xenobiotic or metabolite produced, or by the detoxifying action of GST on oxidative products generated by the DBP poisoning. The fact that we observed a significant increase in the GST activity in muscle in the 2 and 3-μg L⁻¹ groups, but not for 4 and 5 μg L⁻¹, suggests the existence of more than one metabolic pathway associated with the detoxification by GST in electric fish muscle. Although these alternative mechanisms are presently only hypothetical, a similar type of response has been observed when different species of electric fish were exposed to increasing concentrations of different chemicals. Typically, the physiological effects of extraneous compounds on the EOD patterns within the first two hours of exposure tend to be more conspicuous and statistically significant with intermediate than with higher test concentrations. This pattern was observed for formation water, a byproduct of the oil-drilling industry (Rossoni 2005), for neuroactive drugs (Jesus et al. 2017) and for polluted urban effluents (Nunes 2016). Thus, further studies are necessary to elucidate these patterns in electric fish.

Although we observed no significant changes in AChE activity, in Cyprinus carpio exposed to 2 μg L⁻¹ of deltamethrin a 20% decrease in blood serum AChE activity was reported (Balint et al. 1995). Szegletes et al. (1995) also found no changes in AChE activity in C. carpio exposed to a concentration of deltamethrin equivalent to 2 μg L⁻¹, however, they observed a variation in the distribution of AChE molecular forms, indicating that the pesticide can act on AChE even without affecting its activity. Deltamethrin can affect the acetylcholine release in the hippocampus, which could induce a compensatory mechanism of stimulation of AChE to perform an adaptive up-regulation on acetylcholine hydrolysis (Hossain et al. 2004). However, no direct effect of deltamethrin on AChE activity was observed in rat brains, but observed increased activity of HACU (high-affinity choline uptake) (Hossain et al. 2005). HACU is a Na⁺ dependent choline uptake mechanism which maintains choline regulation in cholinergic neurons and, hence, regulates the acetylcholine synthesis performed by choline acetyltransferase, showing that deltamethrin is related to cholinergic processes, but not directly to AChE enzyme activity (Hossain et al. 2005). In the light of the available data, the interaction between pyrethroids and acetylcholinesterase remains unclear and should be further investigated.

CONCLUSIONS

Microsternarchus cf. bilineatus was shown to be highly sensitive to the DBP tested, with a LC₅₀ 96h of 2.15 μg L⁻¹, the lowest concentration reported for an Amazonian teleost to date. AChE and hepatic GST were not considered good.
biochemical markers for *M. cf. bilineatus* exposed to DBP as they were not significantly affected by the tested DBP concentrations and exposure periods. On the other hand, muscle GST increased significantly in fishes submitted to DBP concentrations of 2 and 3 μg L⁻¹, probably as a mechanism for pollutant elimination in this tissue, by xenobiotic detoxification through conjugation, or by the action of GST on products generated by oxidative stress. The lack of increase of GST activity for the higher concentrations (4 and 5 μg L⁻¹), may owe to the existence of more than one internal pathway to deal with physiological and biochemical stress in this species, possibly in association with exposition time. Our findings raise interesting questions regarding the action of pyrethroids on other physiological mechanisms of this electric fish species. For instance, the effect of DBP on the physiology of the electric organ, since electrogenic cells are rich in Na⁺ channels and the regular functioning of these channels controls the passage of electrical impulses that will generate the EODs in these fishes.

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