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Factors that alter the biochemical biomarkers of environmental contamination in *Chironomus sancticaroli* (Diptera, Chironomidae)

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ABSTRACT

Changes in physiology of the nervous system and metabolism can be detected through the activity of acetylcholinesterase (AChE), alpha esterase (EST-α) and beta esterase (EST-β) in chironomids exposed to pollutants. However, to understand the real effect of xenobiotics on organisms, it is important to investigate how certain factors can interfere with enzyme activity. We investigated the effects of different temperatures, food stress and two steps of the enzymatic protocol on the activity of AChE, EST-α and EST-β in *Chironomus sancticaroli*. In experiment of thermal stress individuals from the egg stage to the fourth larval instar were exposed to different temperatures (20, 25 and 30 °C). In food stress experiment, larvae were reared until IV instar in a standard setting (25 °C and 0.9 g weekly ration), but from fourth instar on they were divided into groups and offered different feeding regimes (24, 48 and 72 h with/without food). In sample freezing experiment, a group of samples was processed immediately after homogenization and another after freezing for 30 days. To test the effect of centrifugation on samples, enzyme activity was quantified from centrifuged and non-centrifuged samples. The activity of each enzyme reached an optimum at a different temperature. The absence of food triggered different changes in enzyme activity depending on the period of starvation. Freezing and centrifugation of the samples significantly reduced the activity of three enzymes. Based on these results we conclude that the four factors studied had an influence on AChE, EST-α and EST-β and this influence should be considered in ecotoxicological approaches.

Introduction

Biochemical biomarker responses enable detection of the first biological effects associated with exposure to xenobiotics, even at low concentrations (Lionetto et al., 2003). The enzyme AChE is widely used as a biomarker of exposure to organophosphorate and carbamates compounds, which inhibit this enzyme, thus compromising the nervous system of organisms (Fulton and Key, 2001; Galloway and Handy, 2003). The metabolic enzymes EST-α and EST-β bind to xenobiotics and transform them into a more hydrosoluble compounds facilitating their excretion (Hemingway and Ranson, 2000).

However, prior to using the enzymes AChE, EST-α and EST-β as biomarkers, it is necessary to investigate whether certain factors can change their activity. Organisms in the natural environment face adverse situations on a daily basis, for instance fluctuations in temperature and food availability. In laboratory studies, acute toxicity bioassays are usually performed in the absence of food, which can lead to metabolic stress. Studies using different bioindicators organisms (copepods, crustaceans and bivalves) have investigated the influence of seasonal variations on selected biochemical biomarkers (AChE, glutathione S-transferase, catalase, metallothionein) and their correlation with seasonal fluctuations in abiotic parameters such as temperature, salinity, turbidity and food availability (Leiniö and Lehtonen, 2005; Pfeifer et al., 2005; Menezes et al., 2006; Cailleaud et al., 2007; Tu et al., 2012).

In addition to environmental variations, the effects of laboratory protocols that aim to quantify enzymatic activity need to be standardized for the bioindicator species. Some steps of the protocol, for example centrifugation and freezing of samples, can influence the enzymatic analysis of the biochemical biomarkers (Guilhermino et al., 1996; Murias et al., 2005).

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Immature Chironomidae (Diptera) inhabit the benthic compartment of aquatic ecosystems (Lagauzère et al., 2009; Di Veroli et al., 2012a). They are important components of the food chain, representing the strongest link between producers and secondary consumers (Porinchu and MacDonald, 2003). Because they are sensitive to various pollutants (Preston 2002), are easy to rear and have a short lifespan (Fonseca and Rocha, 2004), chironomids are widely used as bioindicators of acute and chronic toxicity in contaminated sediments and water (Lee et al., 2006; Roulier et al., 2008; Yoshimi et al., 2009; Al-Shami et al., 2010; De Jonge et al., 2012; Di Veroli et al., 2012b; Ebau et al., 2012; Choung et al., 2013). *Chironomus sancticaroli* Strixino and Strixino, 1981 is a well-known bioindicator of water quality, and has been used in various biochemical studies involving biomarkers, in an attempt to elucidate its responses to environmental contamination (Moreira-Santos et al., 2005; Printes et al., 2007, 2011).

The aim of this study was to investigate experimentally the potential effects of food and thermal stress on the activity of the enzymes AChE, EST-α and EST-β of *C. sancticaroli* larvae. In addition, the effects of two steps of the enzymatic protocol (freezing and centrifugation of samples) on enzymatic activity were assessed in order to standardize the methodology.

Material and methods

Biological material

Specimens were obtained from the Laboratory of the Medical and Veterinary Entomology, Federal University of Paraná (UFPR). Their breeding colony is maintained following Maier et al's protocol (1990), with modifications in the temperature (25 °C ± 2) and photoperiod (12 h light:12 h dark). Voucher specimens are deposited in the Pe. Jesus Santiago Moure Entomological Collection of the Department of Zoology, UFPR (DZUP), numbers 249269 to 249276.

Enzymatic assay

Larvae were stored in a −80 °C freezer and were subsequently homogenized in 300 μL 0.1 M pH 7.5 potassium phosphate buffer (for the enzyme AChE) and in 150 μL 0.2 M pH 7.2 potassium phosphate buffer (for the enzymes EST-α and EST-β), followed by centrifugation at 12,000 × *g* for 1 min at 4 °C.

The protocol used for the enzyme AChE was based on Ellman et al. (1961), modified for microplates following Silva de Assis (1998). The activities of the EST-α and EST-β were ascertained following the methodology of Valle et al. (2006). Total protein per larva was measured following Bradford (1976), using bovine serum albumin as standard. Biochemical analyses were carried out in a BioTek microplate reader.

Temperature effects on the larvae

From hatching up to the fourth instar, different groups of larvae were kept at three different temperatures: 20 °C, 25 °C and 30 °C. The temperature was controlled in a BOD constant temperature chamber (photoperiod 12/12 h). The larvae were subsequently subjected to the enzymatic quantification protocols already described. A total of 270 larvae (90 larvae for each enzyme, 30 larvae for each temperature) were used. In this experiment, the effect of temperature on larval development duration was also ascertained.

The effect of food stress on larvae

Stock larvae of *C. sancticaroli* in the IV instar were subjected to six different treatments. In treatments A, B and C, 4 mg TetraMin® per larva were offered at time 0. After 24 h, treatment A was discontinued, followed by treatment B after 48 h and treatment C after 72 h. Larvae in the remaining three treatments, D, E and F, were not fed at time zero and were maintained without food for 24, 48 and 72 h, respectively. Results from the feeding and food deprivation treatments were then compared for the same time periods (A with D, B with E and C with F). This experiment was carried out in containers with 80 mL of dechlorinated water. Larvae were isolated from one another to prevent predation. The treatments were performed in a BOD chamber with constant temperature (25 °C ± 2 °C) and photoperiod (12 h light/12 h dark). In total, 540 IV instar larvae (180 larvae for each enzyme, 30 larvae for each treatment) were processed.

Effects of freezing on homogenized samples

Stock larvae were homogenized as described above for enzyme activity quantification. However, the volume of each sample was divided into two aliquots. One was used immediately for enzyme quantification, while the other was frozen in −80 °C for 30 days before it was used for this purpose. A total of 90 IV instar larvae (30 larvae for each enzyme) were processed.

Effects of centrifugation on homogenized samples

Stock larvae were homogenized as described above for each enzyme. However, the volume of each sample was divided into two aliquots. One was centrifuged, while the other was not. Both aliquots were subjected to enzyme quantification. A total of 90 IV instar larvae (30 larvae for each enzyme) were processed.

Statistical analysis

Analyses were performed in R environment (R Development Core Team, 2011). The effects of temperature on the activity of the enzymes AChE and EST-β were analyzed with an adjusted generalized linear model (GLM) with Gamma distribution, and for the enzyme EST-α, an inverse Gaussian distribution was employed. One way ANOVA was applied, and Tukey contrast ($p \leq 0.05$) was used in *a posteriori* comparisons. MASS (Venables and Ripley, 2002) and effects (Fox, 2003) libraries were used for GLM and the multcomp library was used in *posteriori* analyses (Hothorn et al., 2008). To evaluate the effect of centrifugation and freezing on enzyme activity, data were logaritmised and the t test for paired samples was used. In the analysis of food stress on enzyme activity, data were also logaritmised, but a t test for unpaired samples was applied instead.

Results

Increments of five-degree Celsius during the development of *C. sancticaroli* shortened the development time of immatures from twelve days at 20 °C, to seven days at 25 °C, and to four days at 30 °C. The enzyme activity changed under different temperatures (Fig. 1). AChE activity decreased with increasing temperatures: at 20 °C and 25 °C it was 69% and 59% lower than at 30 °C, respectively. No significant changes in enzyme activity were detected between 20 °C and 25 °C.

No changes in the activity of EST-α were observed between 20 °C and 25 °C (Fig. 1). However, at 30 °C the enzyme activity increased by 44% and 45% when compared to 20 °C and 25 °C, respectively.

The enzyme activity of EST-β was high at the intermediate temperature of 25 °C. At this temperature, EST-β activity was 24% higher than at 20 °C and 18% higher than at 30 °C. In contrast, enzyme activity at 20 °C and 30 °C did not differ (Fig. 1).