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Factors that alter the biochemical biomarkers of environmental contamination in *Chironomus sancticarloi* (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 sancticarloi*. 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.

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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 organophosphate and carbamate 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 (Porinchi 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 sancticarloi* 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. sancticarloi* 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 ($25\text{ }^{\circ}\text{C} \pm 2$) 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 $-80\text{ }^{\circ}\text{C}$ freezer and were subsequently homogenized in $300\text{ }\mu\text{L}$ 0.1 M pH 7.5 potassium phosphate buffer (for the enzyme AChE) and in $150\text{ }\mu\text{L}$ 0.2 M pH 7.2 potassium phosphate buffer (for the enzymes EST- α and EST- β), followed by centrifugation at $12,000 \times g$ for 1 min at $4\text{ }^{\circ}\text{C}$.

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: $20\text{ }^{\circ}\text{C}$, $25\text{ }^{\circ}\text{C}$ and $30\text{ }^{\circ}\text{C}$. 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. sancticarloi* 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 ($25\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$) 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 $-80\text{ }^{\circ}\text{C}$ 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 *a posteriori* analyses (Hothorn et al., 2008). To evaluate the effect of centrifugation and freezing on enzyme activity, data were logarithmised and the t test for paired samples was used. In the analysis of food stress on enzyme activity, data were also logarithmised, but a t test for unpaired samples was applied instead.

Results

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

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

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

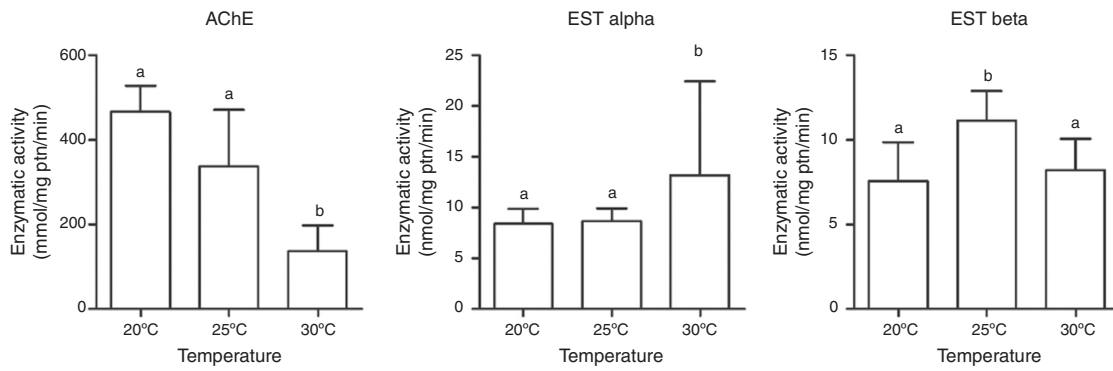


Fig. 1. Effect of temperature (20, 25 and 30 °C) on the activity of acetylcholinesterase (AChE), alpha esterase (EST- α), and beta alpha esterase (EST- β) of *Chironomus sancticaroli*. The values are expressed as the mean value of enzyme activity \pm SD ($n = 30$ for each condition). Different letters indicate significant differences when $p < 0.05$ (using ANOVA – one way and Tukey contrast).

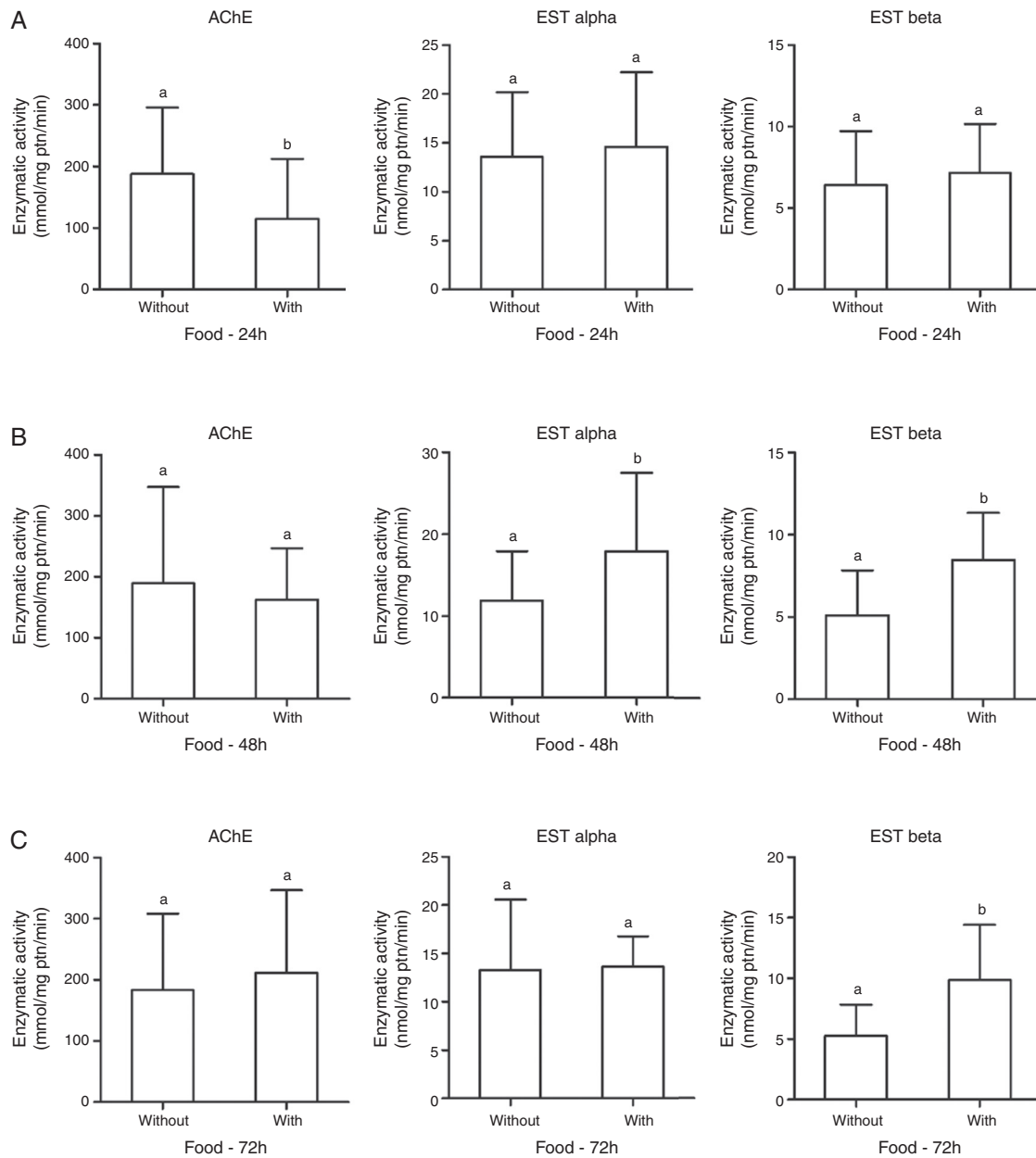


Fig. 2. Effect of fasting for 24 h (A); 48 h (B) and 72 h (C) on the activity of acetylcholinesterase (AChE), alpha esterase (EST- α), and beta alpha esterase (EST- β) of *Chironomus sancticaroli*. The values are expressed as the mean value of enzyme activity \pm SD ($n = 30$ for each condition). Different letters indicate significant differences when $p < 0.05$ (using unpaired t -test).

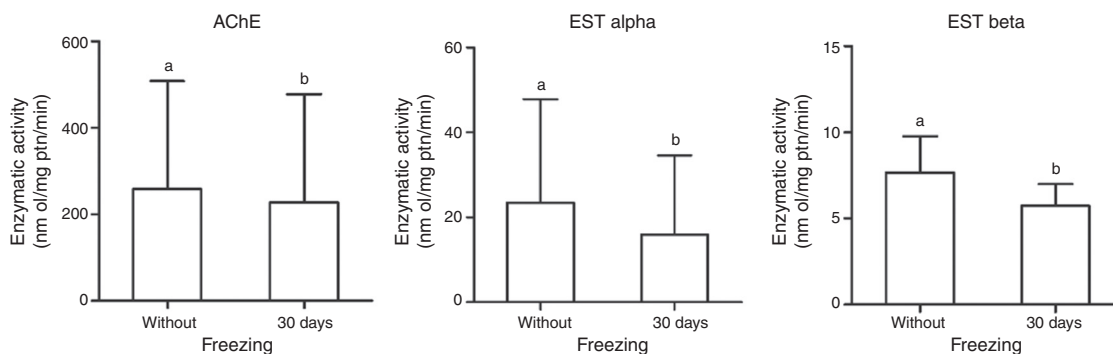


Fig. 3. Effect of sample freezing on the activity of acetylcholinesterase (AChE), alpha esterase (EST- α), and beta alpha esterase (EST- β) of *Chironomus sanctificaroli*. The values are expressed as the mean value of enzyme activity \pm SD ($n = 30$ for each condition). Different letters indicate significant differences when $p < 0.05$ (using paired t -test).

After 24 h of food deprivation, activity of the AChE enzyme increased significantly (39%), while activity of the EST- α and EST- β did not (Fig. 2A). AChE activity did not change after 48 h of food deprivation. Activity of the EST- α and EST- β enzymes was significantly lower after 48 h of food deprivation (34% and 41%, respectively) (Fig. 2B). After 72 h, AChE and EST- α activity remained constant, whereas EST- β activity was significantly reduced by 47% (Fig. 2C).

The results of the freezing and centrifugation tests indicate that these two factors may negatively influence the activity of the three enzymes evaluated. Freezing samples for 30 days at -80°C decreased enzymatic activity of the AChE, EST- α and EST- β by 12%, 32% and 25%, respectively (Fig. 3). Centrifugation of samples also affected the activity of the AChE, EST- α and EST- β : when samples were centrifuged, enzyme activity decreased by 18%, 10% and 10%, respectively (Fig. 4).

Discussion

It is important to investigate how certain factors such as temperature and food resources affect the activity of biochemical biomarkers used to assess the effect of pollutants. A temperature increase during larval development shortens the development period of insects, thus impacting the final size of the adults (Vogt et al., 2007; Oetken et al., 2009; Zilli et al., 2009). This is an indication that temperature can be a metabolic stressor. Park and Kwak (2014) investigated the effects of thermal stress on the development of *Chironomus riparius* Meigen, 1804, showing that it alters the biology (larval survival rate, sex ratio, successful pupation and adult emergence), metabolism (increased expression gene related to oxidative stress enzymes (catalase, peroxidase, superoxide dismutase and glutathione peroxidase) and endocrine signaling (ecdysone receptor) of the organisms.

The effects of temperature on biochemical biomarkers such as the AChE enzyme of invertebrates have been investigated, and the results of various studies varied according to the species. For instance, activity of this enzyme may increase or decrease as temperature increases (Scaps and Borot, 2000; Callaghan et al., 2002; Pfeifer et al., 2005; Menezes et al., 2006; Cailleaud et al., 2007; Tu et al., 2012). In this study, AChE activity decreased at higher temperatures, corroborating the results of Domingues et al. (2007), who observed that the activity of the AChE of *C. riparius* Meigen, 1804 is higher at 6°C and 16°C than at 26°C .

The fact that each enzyme behaves differently under various temperature regimes highlights the fact that each enzyme has an optimum temperature activity (Callaghan et al., 2002). This abiotic factor alters the physical structure of enzymes, and modifies their catalytic efficacy or binding capacity (Hochachka and Somero, 1984). Therefore, when enzymes are used as biomarkers of environmental contamination in aquatic ecosystems, temperature must be taken into consideration and enzymatic activity can only be compared among specimens from similar temperature ranges. Additionally, seasonal variations in temperature must also be considered in analyses.

The effects of food stress on biochemical markers, which have been studied only sporadically, are not well understood. In our data, the lack of food affected the activity of the three tested enzymes (AChE, EST- α and EST- β) differently, according to the period of starvation. After a study using *C. riparius*, Crane et al. (2002) found no differences in AChE activity after 48 and 96 h of food deprivation, although the dry weight of individuals decreased. Studies using other biomarkers, such as fish, indicated that food stress is associated with changes in enzymatic biomarkers, and caused oxidative stress in individuals (Pascual et al., 2003).

In an experiment using *C. riparius*, individuals that were given enough food were less susceptible to pollutants than those that

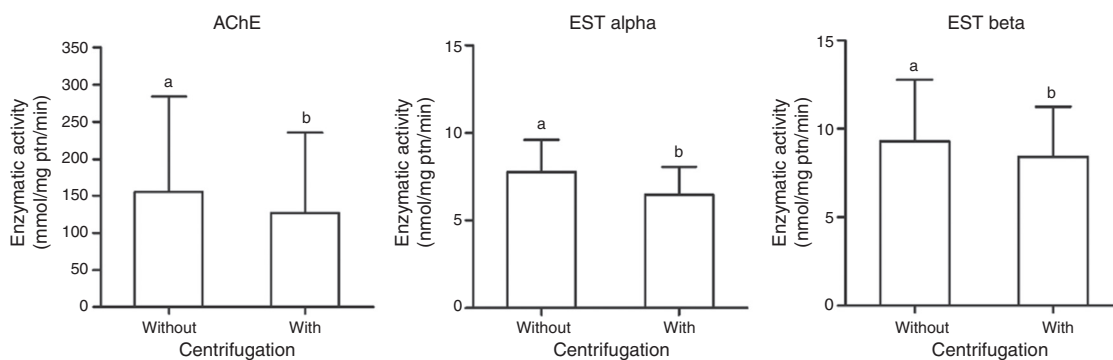


Fig. 4. Effect of sample centrifugation on the activity of acetylcholinesterase (AChE), alpha esterase (EST- α), and beta alpha esterase (EST- β) of *Chironomus sanctificaroli*. The values are expressed as the mean value of enzyme activity \pm SD ($n = 30$ for each condition). Different letters indicate significant differences when $p < 0.05$ (using paired t -test).

were not (Postma et al., 1994). However, an opposite effect can be achieved when food is used in toxicological experiments, increasing the toxicity of certain compounds, for instance cadmium, which quickly binds to organic materials, such as carbon-based compounds derived from food degradation in the experiment (Postma et al., 1994).

Sample freezing after homogenization has been previously investigated and can be part of laboratorial routine when the number of samples is large. In our data, it was evident that freezing lowers enzyme activity. This was expected, as a tendency to decreased enzyme activity after each cycle of freeze-thaw had been previously documented (Murias et al., 2005). Consequently, in laboratorial routine, it is best to homogenize samples and perform enzymatic quantification on the same day as a means to achieve maximum enzyme activity response. This can be difficult sometimes, particularly when the number of samples is large, and the only alternative is freezing. When freezing becomes necessary, we emphasize that samples that will be analyzed together should be processed on the same day and be subjected to the same number of freeze-thaw cycles, thus minimizing the variations introduced by this step.

Another protocol step analyzed in this work was centrifugation, which also reduced enzymatic activity of the samples. This happens because a portion of the enzymes can be removed from the supernatant during centrifugation, as enzymes remain attached to larger fragments that deposit during this process (Guilhermino et al., 1996).

Even though centrifugation causes a negative effect on enzyme activity, this procedure should be used in all protocols, because it purifies the samples for enzymatic quantification, reducing the interference of residues in absorbance readings.

Conclusions

The activity of the enzymes AChE, EST- α and EST- β decreased after freezing and centrifugation of samples, demonstrating the importance of standardized protocols. Additionally thermal and food stress caused changes in the activity of the three enzymes. Based on these results we recommend that temperature and food supply should be maintained constant in toxicity bioassay tests.

Conflicts of interest

The authors declare no conflicts of interest.

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