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Acute toxicity of organophosphate fenitrothion on biomarkers in prawn *Palaemonetes argentinus* (Crustacea: Palaemonidae)

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Abstract The effect of the organophosphate fenitrothion (FS) on the non-target freshwater prawn *Palaemonetes argentinus* was studied. Initially, the 96-h lethal concentration (LC_{50}) of FS was determined in adult prawns. Inhibition of cholinesterase (ChE) in the muscle and hemolymph was assessed. Then, in the hepatopancreas, the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione-*S*-transferase (GST) were analyzed. Lipid peroxidation (LPO) was also determined in the hepatopancreas. The 96-h LC_{50} value was 1.12 μg/L. Hemolymph ChE activity showed a significant decrease in exposed prawns to FS compared to the control group, while no significant differences in the muscle were observed between groups ($p<0.05$). FS caused a significant increase in the activity of antioxidant enzymes SOD, CAT, and GST compared to the control group ($p<0.02$). By contrast, LPO levels were not affected by the pesticide ($p<0.05$). These results indicate that *P. argentinus* is very sensitive to organophosphorus which alter biochemical parameters that are related to antioxidant status. Thus, these parameters could be used as biomarkers for assessing water pollution.

Keywords Biomarker · Fenitrothion · Organophosphorus · Oxidative stress · Prawn · Toxicity

Introduction

Anticholinesterases such as organophosphorus (OPs) are one of the most commonly used pesticides for insect pest management. Despite the high efficiency, the widespread use of OPs results in the release of their residues into natural water, tending to be toxic to non-target organisms such as fish and many species of aquatic invertebrates (Liu et al. 2012). Fenitrothion (FS [*O*,*O*-dimethyl-*O*-(3-methyl-4-nitrophenyl) phosphorothioate]) is an OP that has several applications in agriculture. FS is employed to control insects on rice, cereals, fruits, vegetables, stored grains, and cotton (Zayed and Mahdy 2008). Also, it is currently used for mosquito control (Delatte et al. 2008). Its production is estimated around 15,000–20,000 tonnes/year (Sanchiricoa et al. 2012). Although FS is extremely labile under experimental conditions, field doses may be higher than 500 g/ha and may reach concentrations of 80 μg/L in the water body (Leboulanger et al. 2011). It is known that FS in water solution undergoes photodegradation, resulting in the release of several toxic metabolites, some of which are more toxic to aquatic organisms than the parent compound (Zayed and Mahdy 2008). FS, like other OPs, is bio-activated by the mixed-function oxygenase

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system (MFO) which catalyzes the formation of oxon metabolites (Damasio et al. 2007). These metabolites are effective inhibitors of cholinesterase (ChE); they cause neurotoxic effects due to the accumulation of the neurotransmitter acetylcholine in cholinergic synapses of organisms (Leelaja and Rajini 2013). ChEs are typically subdivided into two major classes, acetylcholinesterases (AChE; EC 3.1.1.7) and butyrylcholinesterases (BChE; EC 3.1.1.8). In aquatic organisms, there is considerable diversity in the biochemical properties and distribution of both enzymes as well as their sensitivity to anticholinesterase agents (Vioque-Fernández et al. 2007). The primary physiological role of BChE is not completely clear, but some studies suggest that this enzyme may play an important role in the hydrolysis of acetylcholine at cholinergic synapses like AChE (Kristoff et al. 2012). Also, BChE could play a protective role in anticholinesterase intoxication by removing the pesticide prior to reaching the target site AChE and contribute to the sensitivity of the species to anticholinesterase pesticides (Sanchez-Hernandez 2007). A number of studies have been performed to urge ChE as a biomarker for prediction of OPs in aquatic invertebrates (Day and Scott 1990; Monserrat et al. 1997; Chang et al. 2006; Frasco et al. 2006; Liu et al. 2012; Rivadeneira et al. 2013). Anyway, substrate preference and tissue distribution of ChE activities in the species selected as a bioindicator should be studied before they are used as biomarkers of OP exposure (Kristoff et al. 2012). Likewise, it is known that OPs increase other biomarkers like activities of antioxidant enzymes: superoxide dismutase (SOD; EC 1.15.1.1) and catalase (CAT; EC 1.11.1.6) (Karami-Mohajeri and Abdollahi 2010). SOD converts $O_2^{\cdot-}$ to H_2O_2, while CAT is an essential enzyme to promote the degradation of H_2O_2, a precursor of the hydroxyl radical that induces DNA damage, protein degradation, and lipid peroxidation (LPO) (Di Giulio et al. 1995). On the other hand, glutathione *S*-transferase (GST; EC 2.5.1.18) is the predominant pathway in most tissues for protection against oxidative stress by conjugating electrophilic compounds with reduced glutathione (Petersen and Doorn 2004). Therefore, GST is another potential biomarker that could predict OP exposure in aquatic invertebrates (Lee et al. 2006).

It has been observed that penaeid prawns are generally more sensitive than fish and mollusks to most pesticides. They have been proposed as indicators of estuarine health due to their worldwide distribution (García-de la Parra et al. 2006). Palaemonetes represent a diverse group of fresh and brackish-water prawns as they are ecologically important as a detritivore, predator of small invertebrates, and prey for fish and birds (Chiodi Boudet et al. 2013). Some species of the genus have been proposed as bioindicators of anthropogenic impact due to their sensitivity to toxicants and other advantages such as easy collection, handling, and laboratory tests (Key et al. 2006). *Palaemonetes argentinus* (Nobili 1901) is a species of ecological interest because it is an abundant non-migratory freshwater prawn in Pampean region where agricultural activity is intensive (Montagna and Collins 2007). Pampasic streams are strongly influenced by pesticides due to the fact that they are largely used because Argentina is a major food exporter in developing countries (Mugni et al. 2011).

Although several aspects of *P. argentinus* have been studied, data regarding toxicology is scarce (Rodrigues Capítulo 1984; Collins and Capello 2006; Montagna and Collins 2007; Chiodi Boudet et al. 2013), especially with respect to research exploring the potential biomarkers in this species to evaluate the use of insecticides in the region. The aim of the present work was: (a) to determine the lethal concentration (LC_{50}) of FS on the freshwater prawn *P. argentinus* and (b) to evaluate the effects of FS in this species using a variety of potential biomarkers including ChE, SOD, CAT, and GST activities and LPO levels.

Materials and methods

Sample collection

P. argentinus adults were collected in a watercourse close to La Plata River, Argentina (20 km SW from La Plata city) in late winter during the pre-reproductive season. They were taken to the laboratory and kept in dechlorinated tap water ($CaCO_3$ hardness, 160 mg/L, pH between 6.6 and 6.9, and dissolved oxygen between 4.5 and 5 mg/L) at 22±2 °C and 14:10-h L:D photoperiod for at least a week before the experiments.

Determination of LC_{50} values

For the experiments, prawns at the intermolt stage according to setogenesis were selected (Drach and Tchernigovtzeff 1967) and then randomly placed at FS concentrations (Glex s, formulated, which was 20 % of active principle purchased from GLEBA S.A. La Plata,

Argentina) and the control. A stock solution of 100 g/L FS was prepared in absolute ethanol (grade p.a.). The subsequent working solutions were obtained by diluting the main stock in absolute ethanol to the following concentrations for exposures: 0.3, 0.6, 0.9, 1.2, 1.8, 2.4, and 4.8 μg/L FS (the final concentration of ethanol was always lower than 0.0001 %), using Triton X-100 as another solvent (final concentration 8 μg/L) as described by González- Baró et al. (1997) plus the control group held without FS but with ethanol and Triton X-100 and another water control group (Lavarías et al. 2013).

Six adult prawns (mean weight 0.7 g) were placed in a glass aquarium containing 2 L of test solution. Two aquaria were used for each treatment (12 animals per treatment).

Because FS could be extremely labile in experimental conditions (Leboulanger et al. 2011), its concentrations were measured according to the EPA method 3510C by gas chromatography (Agilent 6890 equipped with a nitrogen-phosphorous detector), using a capillary column (Hewlett-Packard 5 % phenyl methyl siloxane) of 30 m × 0.25 mm and 0.25-mm film thickness. Helium was used as a carrier and nitrogen as an auxiliary gas. As the effective concentration of FS at the beginning of exposure was reduced to 48.2±2.6 % after 24 h, the test solution was replaced daily. Mortality was recorded and dead prawns were removed every 24 h. Temperature, pH, and dissolved oxygen were measured in all containers. Experiments were done in triplicate at 20–22 °C and a 14-h light:10-h dark cycle with constant aeration. The prawns were not fed for 2 days before the assay and during the exposure period. After the 96-h assays, live prawns were collected for subsequent analysis of biomarkers.

Preparation of total cellular homogenate

After exposure to FS, the prawns were anesthetized on ice for about 5 min, and the hepatopancreas and muscle were dissected, weighed, cooled on ice, and then stored at −70 °C until used.

The hepatopancreas were pooled (two prawns each) and homogenized (1:9 *w*/*v*) in 125 mM Tris-base cold buffer solution, pH 6.8, containing 1 mM 2-mercaptoethanol and 0.1 mM PMSF (Vijayavel et al. 2004). Homogenates were centrifuged at 10,000 × *g* at 4 °C for 10 min and the supernatant used for determining antioxidant enzyme activities and LPO levels. Muscle from each prawn was homogenized as the same hepatopancreas described above (1:5 *w*/*v*) and the supernatant used for determining cholinesterase activity. Total protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Hemolymph extraction

Hemolymph was aseptically withdrawn from the pericardial sinus using a 1-mL syringe with a 1.5-in., 23-gauge needle rinsed with crustacean anticoagulant solution (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, and 20 mM EDTA, pH 4.5) (Sierra et al. 2001). Hemolymph samples were pooled (three specimens each) and centrifuged at 10,000 × *g* for 10 min at 4 °C to remove hemocytes; the resulting supernatant was stored at −70 °C for cholinesterase assay.

Cholinesterase activity

The total activity of cholinesterase (ChE) was determined according to the modified version of the colorimetric technique described by Ellman et al. (1961). The reaction mixture was prepared in 50 mM of potassium phosphate buffer, pH 7.7, containing butyrylthiocholine iodide and 5,5′ dithiobis-2-nitrobenzoic acid at final concentrations of 6 and 0.25 mM, respectively. Ten microliters of hemolymph or muscle supernatant was added to start the reaction, which was followed spectrophotometrically at 405 nm and 25 °C. ChE activity was expressed as enzyme units per milligram of protein. One ChE unit was the amount of enzyme that hydrolyzed 1 nmol of butyrylthiocholine per minute.

Total protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Antioxidant enzyme analyses

SOD activity was assayed according to Misra and Fridovich (1972). The inhibitory effect of SOD on the auto-oxidation of epinephrine (60 mM, pH 2) in 50 mM glycine buffer (pH 10.2) was analyzed spectrophotometrically at 480 nm. Results were expressed as units of SOD per milligram of protein. One SOD unit was considered as the amount of enzyme necessary to inhibit 50 % rate of autocatalytic adrenochrome formation per minute.

CAT activity was measured by the decrease in absorbance at 240 nm due to H_2O_2 consumption, in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7) and 10 mM H_2O_2 according to Aebi (1984). Results were expressed as picomoles of CAT per milligram of protein. One CAT unit was the amount of enzyme required to catalyze 1 pmol of H_2O_2 degraded per minute.

GST activity was assayed using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate; the final reaction mixture contained 1 mM CDNB and 1 mM reduced glutathione (GSH) as described by Habig et al. (1974). Results were expressed as units of GST per milligram of protein. One GST unit represented the amount of enzyme required to conjugate GSH with 1 μmol of 1-chloro-2,4-dinitrobenzene per minute determined at 340 nm.

Lipid oxidation

The LPO level was quantified through the method of Buege and Aust (1978) by measuring thiobarbituric acid-reactive substances (TBARS). Hepatopancreas homogenates were added to the reaction mixture (trichloroacetic acid 15 % (*w*/*v*), 2-thiobarbituric acid 0.375 % (*w*/*v*), and butylhydroxytoluene 0.147 mM) at a ratio of 1:20 (*v*/*v*). The mixture was vigorously shaken, maintained in boiling water for 60 min, and immediately cooled at 4 °C for 5 min (Ohkawa et al. 1979). Then it was centrifuged at 5000×*g* for 10 min and the supernatant measured spectrophotometrically at 535 nm. LPO was expressed as nanomoles of TBARS hydrolyzed per milligram of wet weight.

Statistical analyses

Lethal concentration (LC_{50}) values and their 95 % confidence limits for 24-h intervals were estimated with the standard method of PROBIT analysis program version 1.5 (US, EPA) as described by Finney (1971). Statistical comparison of different treatments was done using a one-way ANOVA after checking for normality and homogeneity of variances. Results of the biomarker analysis are shown as mean±standard deviation (SD). Significant differences ($p<0.05$) were compared using the Tukey post hoc test. Data were analyzed using Instat v. 3.01.

Results

The toxic effect of FS was evaluated on adult prawns *P. argentinus*. As expected, the sensitivity of the prawns to FS increases with the exposure time. The different values of LC_{50} determined for *P. argentinus* are shown in Table 1. No prawns died in the control groups during the experiment, but mortality started at 24 h to 1.2 μg/L of FS exposure, and there was no survival at 72-h 4.8 μg/L FS exposure. The variation between LC_{50} values at 24 and 96 h was 86.5 % in assay.

ChE activity

The hemolymph of control prawns showed the highest activity of ChE, ten times higher than that observed in the muscle. Hemolymph ChE activity of the exposed prawns for 96 h showed a decrease at three concentrations of FS tested compared to the control ($p<0.0001$): 0.3 μg/L (24 %), 0.6 μg/L (27 %), and 0.9 μg/L (52 %), presenting the greatest inhibition at the highest tested concentration (Fig. 1). In the muscle, the activity of ChE was not affected by any concentration of FS in the treated prawns (Fig. 2a).

Effects of FS on oxidative stress parameters

The effect of FS on the enzymatic antioxidant defense was evaluated in prawn hepatopancreas. Figure 3a shows that SOD activity significantly increased ($p<0.02$) by 61 % only at 0.3 μg/L of FS treatment and there were not any differences compared to control at higher FS concentrations.

CAT activity significantly increased ($p<0.0001$) in the treated organisms as compared with controls due to FS exposure by 77, 100, and 173 % at 0.3, 0.6, and 0.9 μg/L of FS, respectively (Fig. 3b).

GST activity significantly increased ($p<0.0001$) at the highest FS concentrations by 102 and 167 % at 0.6

Table 1 LC_{50} values of adult prawns *P. argentinus* exposed to FS

LC_{50}	FS (μg/L)	Confidence intervals
24-h	8.88	4.0–17.0
48-h	3.59	2.44–8.0
72-h	1.45	1.24–1.72
96-h	1.2	1.04–1.40

Equation of the probit analysis: $y=15.364e^{-0.0288x}$, $R^2=0.9394$

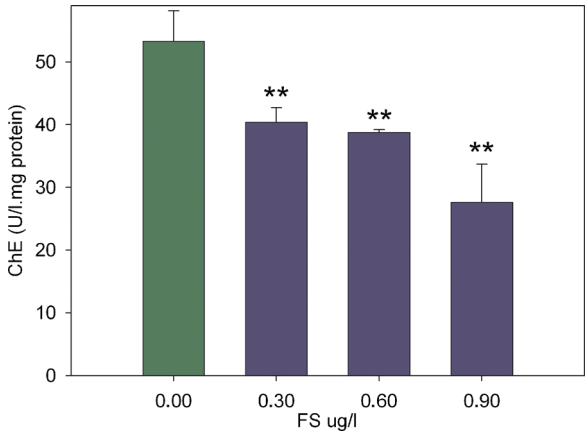

Fig. 1 Effect of different concentrations of FS on hemolymph ChE activity of *P. argentinus*. ChE activity was assayed using butyrylthiocholine iodide as substrate. Each *bar* represents the mean±S.D. of 12 determinations. **Significantly different from the corresponding control ($p<0.0001$)

and 0.9 μg/L of FS, respectively (Fig. 3c). The same behavior observed for CAT was presented by GST; there was a relationship between the increases in activity with increases in FS concentrations.

No significant difference in LPO levels was observed in the prawns exposed to FS compared to the control (Fig. 2).

Discussion

P. argentinus was found to be very sensitive to FS toxicity in comparison with other species within the genus as *P. pausidens* (Takimoto et al. 1987) and *P. varians* (Symons 1976). Moreover, *P. argentinus* is more sensitive than *Macrobrachium borellii* to FS (Lavarías et al. 2013). Both of them coexist in the same ecosystem (Boschi 1981), and they are considered the most frequent and important members of the littoral-benthic community for preying various levels of the food web (Collins and Paggi 1998; Collins 1999). In this context, *P. argentinus* could be proposed as a good autochthonous bioindicator because it is a very sensitive species of crustaceans as proposed by Chiodi Boudet et al. (2013).

It has been established that monitoring ChE activity in estuarine invertebrates is a valuable method for detecting environmental contamination by OPs (Escartin and Porte 1996; Dias Bainy 2000; Crane et al. 2002; Key and Fulton 2002; Devi et al. 2005; Monserrat et al. 2007; Xuereb et al. 2007; Domingues, et al. 2008). This fact is particularly important since many of these chemicals have relatively short half-lives in the aquatic environment and rapid metabolism in biota (Gagnaire et al. 2008; Lacorte et al. 1995). In contrast, after being exposed to OPs, ChE recovery in organisms is very slow. Therefore, enzymatic inhibition can be detected, although there is no longer pesticide in the water, so this biochemical response may offer an advantage in monitoring OPs over the use of only chemical analysis (Rivadeneira et al. 2013). It has been reported that aquatic invertebrates possess more than one active ChE and that these ChEs differ in many aspects from either vertebrate AChE or BChE (Sanchez-Hernandez 2007). For example, in fish, brain and muscle tissues contain mostly AChEs, while the liver and plasma contain mostly BChEs (Habig and Di Giulio 1991). Although, in the muscle of *Procambarus clarkii* exposed to FS, AChE and BChE activities showed a linear correlation (Escartin and Porte 1996), the ChEs of aquatic invertebrates generally show a preference for acetylthiocholine rather than butyrylcholine as substrate (Kristoff et al. 2012). In the case of *P. argentinus*, ChE hemolymph activity was almost ten times higher compared to that of the muscle using the latter substrate. Recent works have suggested that hemolymph ChE activity is considered to be rapid, inexpensive, and a reliable means for measuring the biological impact of pesticide, mainly if sacrificing the animals is avoided, but its activity may not be related directly to nervous system function as gills, muscle, or hepatopancreas. Thus, the enzyme(s) of hemolymph may provide a biomarker of exposure but not necessarily of effect (Brown et al. 2004). While the hemolymph of mussels *Mytilus edulis* showed the highest ChE activity like *P. argentinus*, it was totally insensitive to exposure at OPs (Yaqin and Hansen 2010). In *P. argentinus*, FS treatment affected only the hemolymphatic ChE, while the muscle isoform showed no significant alteration. These findings are similar to those obtained for *M. borellii* (Lavarías et al. 2013). *Litopenaeus stylirostris* treated with FS showed decreased muscle AChE activity while it was not affected in *Penaeus vannamei*. Lignot et al. (1998) explained that the different AChE sensitivity between these prawns can be attributed to the different affinity and phosphorylation rates of AChE. Therefore, ChE activity in invertebrates can be highly divergent (Escartin and Porte 1996; Fulton and key 2001; Key and Fulton 2002; Monserrat et al. 2007; Xuereb et al. 2007; Kristoff et al. 2012), and it is

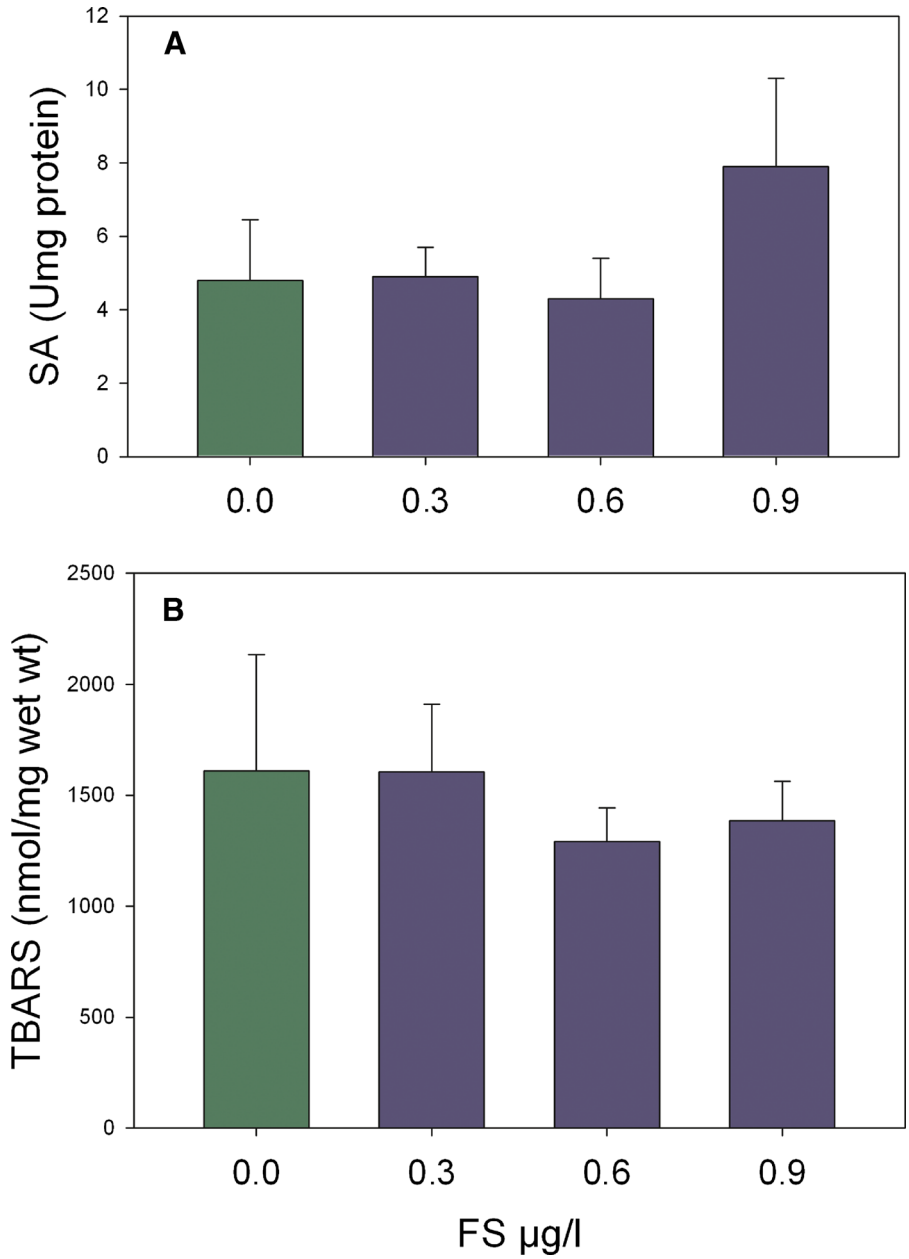

Fig. 2 Effect of different concentrations of FS on the enzymatic activity of muscle ChE (**a**), and lipid oxidation in the hepatopancreas (**b**) of *P. argentinus*. Each *bar* represents the mean±S.D. of 12 determinations

necessary to characterize its activity using different substrates in different tissues to assess this parameter as biomarkers of exposure to OPs.

Although OP mainly affects ChE, the inhibition of other enzymes and the stimulation of oxygen consumption have also been observed after exposure to these pesticides (Day and Scott 1990). Porte and Escartin (1998) demonstrated that the hepatopancreas of the crayfish *P. clarkii* is able to metabolize FS into oxidized products as fenitrooxon after the oxidative biotransformation process by the MFO system, which generates harmful reactive oxygen species (ROS). In fact, there is evidence that the hepatopancreas is the major ROS-producing tissue in crustaceans causing LPO and the induction of antioxidant enzyme activities in this organ by exposure to OPs (Bianchini and Monserrat 2007).

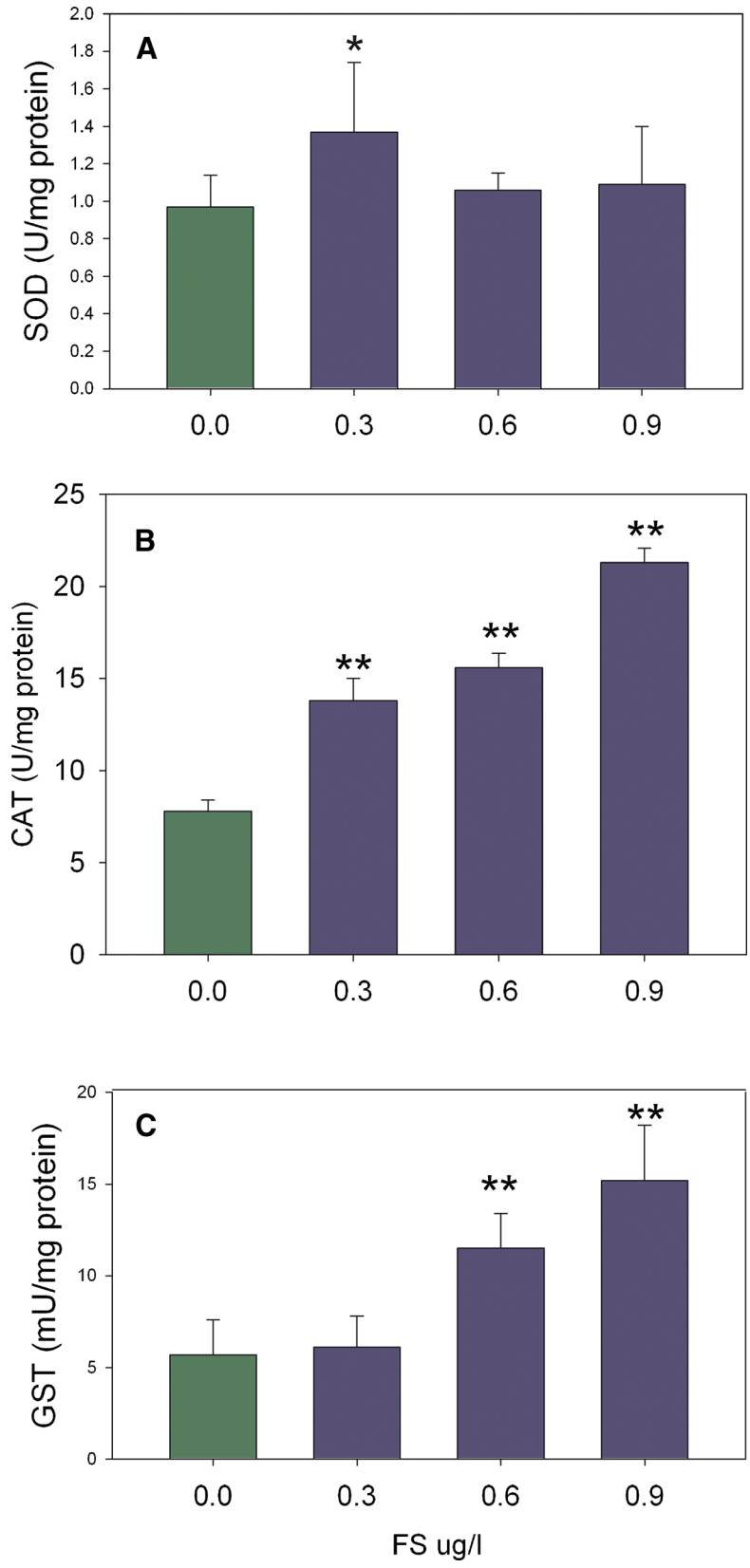

Fig. 3 Effect of different concentrations of FS on the enzymatic activity of superoxide dismutase (**a**), catalase (**b**), and glutathione-*S*-transferase (**c**) in the hepatopancreas of *P. argentinus*. Each *bar* represents the mean±S.D. of 12 determinations. Significantly different from the corresponding control: *$p<0.02$; **$p<0.0001$

However, in *P. argentinus*, levels of LPO in the hepatopancreas were not affected after 4 days of treatment with FS. Oliveira et al. (2013) observed the same effect in prawn *Palaemon serratus* exposed to FS. One possibility is that at the concentrations tested in the present study, the antioxidant enzymatic activities would be enough to remove ROS and avoid LPO. On the other hand, metabolites measured by TBARS, like malondialdehyde, could be metabolized into hydrosoluble molecules which are released into water underestimating LPO (Sukhotin et al. 2002).

Considering the antioxidant defense, the three enzymes assayed showed an increase in hepatopancreas of *P. argentinus* exposed to FS. SOD activity only increased in prawns exposed at the lowest concentration of FS, but at the highest concentrations, no differences with the control were observed. On the other hand, CAT activity was altered in all treated prawns. Therefore, SOD, compared to CAT, could be a very sensitive enzyme presenting inhibition in its activity at higher concentrations of FS. This behavior was observed in *M. borellii* exposed to FS that showed an increase in SOD mRNA expression, while SOD activity was not modified with respect to control, suggesting a compensatory mechanism to overcome the enzymatic inactivation induced by ROS with an increase of enzyme synthesis (Lavarías et al. 2013). Also, in *Macrobrachium rosenbergii* the oxidative stress produced by OP trichlorofon promoted the inhibition of SOD with the resulting accumulation of O_2^- (Chang et al. 2006). So CAT in *P. argentinus* is possibly the main enzyme responsible for avoiding the accumulation of ROS in the organism.

As noted in *P. argentinus*, there is evidence that FS exposure increased GST activity in the hepatopancreas of both *M. borellii* (Lavarías et al. 2013) and the crayfish *P. clarkii* (Blat et al. 1988) due to the fact that GST would be strongly involved in the detoxification of FS products during its metabolization. Indeed, the results in *Daphnia magna* indicated that fenitrooxon was demethylated probably by GST isoforms (Takimoto et al. 1987). Furthermore, metabolization of FS by purified microsomes of the crayfish *P. clarkii* was faster in the GSH- than in the NADPH-supplemented system indicating a great importance of the GST system versus the MFO system as detoxification pathway (Escartin and Porte 1996). On the other hand, *P. serratus* exposed to FS showed alterations in enzymes involved in the production of energy, possibly in an attempt to cope with additional energetic demands; however, CAT and GST activities were not affected (Oliveira et al. 2013).

Results by Graça et al. (2002) showed that the prawn *P. argentinus* could be considered sensitive and, therefore, be used in assays aiming to assess the biological quality of water. The present work clearly shows that in *P. argentinus* the activities of antioxidant enzymes in the hepatopancreas as well as the inhibition of ChE in the hemolymph are more sensible biological variables than those observed in *M. borellii*. Moreover, ChE activity should be further studied in order to be utilized as biomarkers of aquatic pollution by OPs.

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